(51) International Patent Classification 5 :	A2	(11) International Publication Number: WO 91/10140
G01N 33/68, 21/64, 24/08 G07D 233/68, G01D 233/64		(43) International Publication Date: 11 July 1991 (11.07.91)

(21) International Application Number: PCT/CA90/00457

(22) International Filing Date: 28 December 1990 (28.12.90)

(30) Priority data:
458,926   29 December 1989 (29.12.89)   US
577,367   2 May 1990 (02.05.90)   US

(71) Applicant: UNIVERSITY TECHNOLOGIES INTERNATIONAL INC. [CA/CA]; 2500 University Drive, N.W., Calgary, Alberta T2N 1N4 (CA).

(72) Inventors: MOORE, Graham, J. ; 1762 1st Avenue, N.W., Calgary, Alberta T2N OB1 (CA). MATSOUKAS, John, M. ; 29 Saint Dionissios, GR-26223 Patras (GR).

(74) Agent: OSLER, HOSKIN & HARCOURT; Suite 1500, 50 O'Connor Street, Ottawa, Ontario K1P 6L2 (CA).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
   Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR MODELLING TERTIARY STRUCTURES OF BIOLOGICALLY ACTIVE LIGANDS INCLUDING AGONISTS AND ANTAGONISTS THERETO AND NOVEL SYNTHETIC ANTAGONISTS BASED ON ANGIOTENSIN

(57) Abstract

Disclosed are methods f r modelling the three-dimensional structure (tertiary structure) of a ligand having ne or more active sites empl ying a charge-transfer interaction. Also discl sed is a model f r Angiotensin II derived from such method as well as novel synthetic antagonists based on angiotensin.

METHODS FOR MODELLING TERTIARY STRUCTURES OF BIOLOGICALLY ACTIVE LIGANDS INCLUDING AGONISTS AND ANTAGONISTS THERETO AND NOVEL SYNTHETIC ANTAGONISTS BASED ON ANGIOTENSIN

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention is directed to methods for modelling tertiary (three-dimensional) structures of biologically active ligands, to methods for designing and synthesizing agonists and antagonists to the ligands based on the three-dimensional models generated for such ligands, and to the model itself generated for Angiotensin II from the methods of this invention.

2. State of the Art.

In the field of chemistry, compounds can be defined in several ways. For example, a compound can be defined by its empirical formula, e.g., in the case of n-hexane the empirical formula would simply be C_6H_{14}. For simple molecules such as water, methane, carbon dioxide, etc., the empirical formula can provide useful information.

However, as the complexity of the molecule increases, the empirical formula must be complemented by structural information concerning the covalent bonding of the individual atoms vis-à-vis each other in order to derive meaningful information concerning the molecule. Such information is gen rally depicted as a

-2-

the covalent bonds between the respectiv atoms. Such
primary structures are well known pictorial
representations of the compound of interest. These
representations are usually defined as the structural
5   formula of the compound which, for example, in the case
of say n-hexane would be represented as:

```
        H H H H H
      H-C-C-C-C-C-C-H.
        H H H H H
```
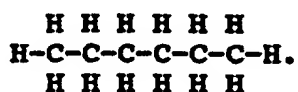
10   However, even with the molecule's structural formula,
valuable information is still missing regarding the
position in three-dimensional space of the individual
atoms relative to each other.   Such three-dimensional
structures or conformations for a molecule are
15   determined in part by non-covalent interactions, e.g.,
electrostatic and non-electrostatic interactions such
as ionic interactions, hydrogen bonding, Van der Waal
forces, etc., between different atoms of the molecule.

Three-dimensional information, i.e., the
20   ligand's conformation, is extremely valuable for
naturally occurring biologically active ligands. In
particular, such biologically active ligands generally
have one or more active sites on or within the
molecular structure of the ligand. Such active sites
25   can involve a charge-transfer interaction (as later
defined). When such a ligand is bound to its
complementary receptor molecule, the active site
activates the receptor molecule thereby affecting the
biological activity of the receptor molecule. Thus,
30   activation of the active site, whether by a charge-
transfer interaction mechanism or by some other
mechanism, is generally a necessary step in affecting
the biological activity f the receptor. Further in

-3-

this regard, if it were possible to create an accurate
three-dimensional model of the naturally occurring
biologically active ligand [including its active
site(s)] as found _in vivo_, then such models could be
5   used to create mimetics, e.g., agonists and
antagonists, of such ligands. For example, if it is
desirable to suppress the biological activity of the
receptor _in vivo_, then an accurate three-dimensional
model of the receptor's naturally occurring
10   complementary ligand including its active site(s),
would greatly facilitate the preparation of antagonists
to this receptor. Likewise, an accurate three-
dimensional model of the ligand of interest would also
facilitate the design and synthesis of agonists when it
15   is desirable to increase or to stimulate the biological
activity of the receptor _in vivo_.

While three-dimensional models have
heretofore been proposed for molecules including
ligands, such three-dimensional representations have
20   suffered from one or more serious drawbacks,
particularly as they relate to biologically active
ligands having active site(s) which employ a charge-
transfer interaction. In particular, such prior art
methods have failed to provide a simple means to
25   identify the active site(s) of such ligands.
Accordingly, in such cases, the creation of a three-
dimensional model of such a ligand including its active
site was generally conducted by extremely laborious
procedures such as structure-activity relationships,
30   theoretical considerations, etc. However, because such
procedures are unable to identify a charge-transfer
interaction at the active site of these ligands, it has

-4-

not been possible to model mimetics of such ligands to a meaningful conformation.

Additionally, other art recognized methods of modelling the tertiary structure of a compound in

5   three-dimensional space, such as x-ray crystallography, have the drawback that with biologically active ligands, the steps required to prepare the ligand for analysis can change the ligand's tertiary structure and accordingly, the structure as determined by this

10   analysis may not conform to the structure found _in vivo_. Moreover, not all biologically active ligands are amenable to such analysis.

In view of the above, it is an object of this invention to develop a process which would model the

15   three-dimensional spatial (tertiary) structure of a biologically active ligand having one or more active sites employing a charge-transfer interaction. It is a further object of this invention that this modelling identify the chemical groups at the site(s) of charge-

20   transfer interactions. It is still a further object of this invention to create models of such ligands closely resembling the structure of the ligand found _in vivo_. It is still another object of this invention to design mimetics to such ligands by reference to the model

25   generated for the ligand. These and other objects are achieved by the present invention as evidenced by the attached summary of the invention, detailed description of the invention, examples and claims.

-5-

SUMMARY OF THE INVENTION

The above objectives are achieved by the methods of the present invention. In particular, by using these methods, one is now able to model
5   biologically active ligands having one or more active site(s) which employ charge-transfer interactions. The methods of the present invention involve identification of a charge-transfer interaction using fluorescent methods, identification of the groups involved in the
10 charge-transfer interaction by structure-activity studies, and application of NMR methods to resolve remaining aspects of the conformation surrounding the charge-transfer interaction. Moreover, the model or conformation so obtained is used in a method to design
15 mimetics, i.e., agonist and antagonists, of such ligands. Accordingly, in one of its method aspects, the present invention is directed to a method for creating a three-dimensional spatial model for a biologically active ligand having one or more active
20 sites based on a charge-transfer interaction and further having a known structural formula wherein the three-dimensional spatial assignments for each of the atoms of the ligand in the model are assigned from the steps comprising:
25   a)   determining the presence of charge-transfer interaction(s) in said ligand from fluorescence analysis of said ligand in a fluorescence compatible environment;
               b)   determining the chemical groups involved
30 in said charge-transfer interaction(s); and
               c)   resolving remaining aspects of the ligand's three-dimensional conformation by obtaining conformational information relative to the active sit (s) from nucl ar magnetic resonanc spectroscopy

-6-

employing the nuclear Overhauser effect providing that
when the nuclear Overhauser effect technique employed
in this step is NOESY, then the molecular weight of
said ligand is either less than about 500 or greater
5   than about 2000.

           Another method aspect of the present
       invention is directed to a method of modelling
       antagonists to a biologically active receptor based on
       the model generated for a biologically active ligand
10   complementary to said receptor wherein said ligand has
       one or more active sites based on a charge-transfer
       interaction and further has a known structural formula
       which method comprises the steps of:
           a)   creating a three-dimensional spatial
15   model for said ligand by
                 i) determining the presence of charge-
       transfer interaction(s) in said ligand from
       fluorescence analysis of said ligand in a fluorescence
       compatible environment;
20   ii) determining the chemical groups
       involved in said charge-transfer interaction(s); and
       iii)resolving remaining aspects of the ligand's three-
       dimensional conformation by obtaining conformational
       information relative to the active site(s) from nuclear
25   magnetic resonance spectroscopy employing the nuclear
       Overhauser effect providing that when the nuclear
       Overhauser effect technique employed in this step is
       NOESY, then the molecular weight of said ligand is
       either less than about 500 or greater than about 2000;
30   and
           b)   identifying a compound having a three-
       dimensional structure sufficiently similar to said
       ligand so as t be complementary to said r ceptor and

-7-

wherein at least one of the charge-transfer
interactions in said compound has been compromised.

Still another method aspect of the present
invention is directed to a method of modelling agonists
to a biologically active receptor based on the model
generated for a biologically active ligand
complementary to said receptor wherein said ligand has
one or more active sites based on a charge-transfer
interaction and further has a known structural formula
which comprises the steps of:

a)   creating a three-dimensional spatial
model for said ligand by

i) determining the presence of charge-
transfer interaction(s) in said ligand from
fluorescence analysis of said ligand in a fluorescence
compatible environment;

ii) determining the chemical groups
involved in said charge-transfer interaction(s); and

iii) resolving remaining aspects of the
ligand's three-dimensional conformation by obtaining
conformational information relative to the active
site(s) from nuclear magnetic resonance spectroscopy
employing the nuclear Overhauser effect providing that
when the nuclear Overhauser effect technique employed
in this step is NOESY, then the molecular weight of
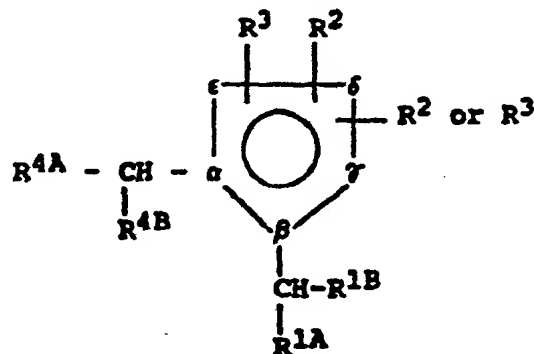said ligand is either less than about 500 or greater
than about 2000; and

b)   identifying a compound having a three-
dimensional structure sufficiently similar to said
ligand so as to be complementary to said receptor and
wherein the charge-transfer interaction(s) in said
compound has (have) not been compromised.

-8-

Yet another method aspect of the present invention is directed to a method for determining the presence of charge-transfer interaction(s) in the tertiary structure of a biologically active ligand
5   complementary to a biologically active receptor which comprises conducting fluorescence analysis of said ligand in a fluorescence compatible environment.

In a preferred embodiment, the above described methods are particularly suitable for
10   modelling a three-dimensional spatial structure of Angiotensin II. FIGURE 6 of this application illustrates a stereo photograph of a molecular model (three-dimensional model) for Angiotensin-II. FIGURE 8A of this application illustrates a stereo photograph
15   of a molecular model (three-dimensional) for receptor-bound Angiotensin II. Accordingly, another aspect of this invention is directed to the model of Angiotensin-II illustrated in FIGURE 6 as well as the model of receptor-bound Angiotensin II illustrated in FIGURE 8A.

20   A product aspect of the present invention is directed to a compound of the formula:
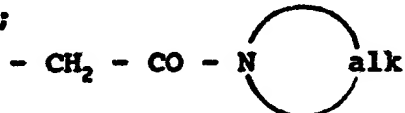
-9-

wherein $\alpha, \beta, \gamma, \delta$ and ϵ are C, N, O or S with the provisos that (a) the ring contains at least one C atom and one N atom, and (b) attachment of R groups is to C or N, and preferably further with the provisos that (c) at least one ring N atom remains unsubstituted, and (d) the pKa of the ring is ≤ 7 when all attendant groups have been taken into account;

R^{1A}, which mimics the structure in angiotensin of $- CH - CO - N - CH - CO -$

includes the following:

-alk; -O-alk; -alk-O-alk; $-CH_2-CO-NH_2$; $-CH_2-CO-NH-alk$; $-CH_2-CO-N(alk)_2$;

$- CH_2 - CO - N \bigcirc alk$

$-CH_2-CO-AA-NH_2$; or $CH_2-CO-AA-Phe$, wherein AA is an amino acid preferably proline, azetidine-carboxylic acid, pipecolic acid, nipecotic acid, glycine, alanine, sarcosine, or N-methyl-alanine;

R^{1B}, which optionally provides a spacer arm terminating in a mimic of the C-terminal carboxylate group of angiotensin II, includes the following:

-H; -alk-A; $-\bigcirc^A$; or $-alk-\bigcirc^A$;

preferably with the proviso that when R^{1B} is H, then: (a) if the ring is imidazole α and/or γ is other than N, (b) if the ring is other than imidazole either α is C or β is N, (c) R^{1A} comprises a group containing an amide, (d) R^2 comprises a group containing A, or (e) R^3 comprises a group containing B or $-Asp-Arg-NH_2$;

R^2, which provides steric and/or electronic properties and/ r a spac r arm terminating in an acid group, includes the following: -H, -halide; -alk;
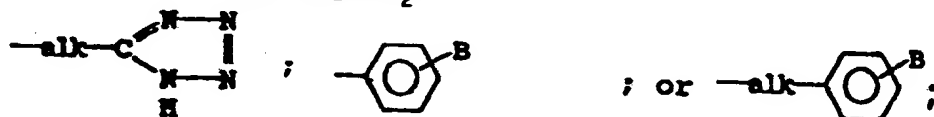
SUBSTITUTE SHEET

-10-

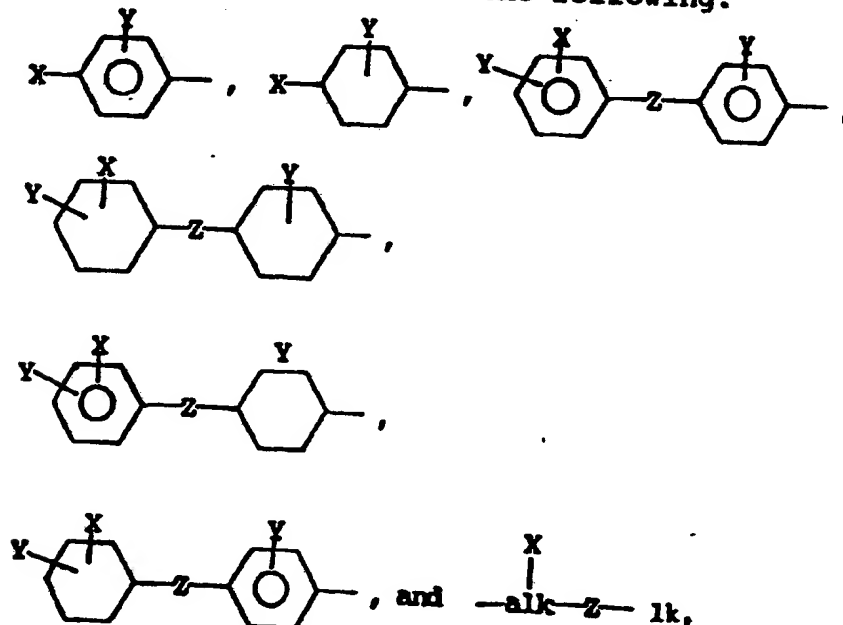-O-alk; -NO$_2$; -CF$_3$; -CN; -alk-A; -A;

 ; or

R^3, which provides steric and/or electronic properties and/or a mimetic of the tyrosine hydroxyl group of angiotensin II in its "charge relay" conformation, or a spacer arm terminating in a mimic of the N-terminus of N-terminal dipeptide of angiotensin-II, includes the following;

-H; -alk; -aryl; -alk-OH; -alk-halide; -CH$_2$-O-alk; -CH$_2$-CN; -CH$_2$-CO$_2$H; -CH$_2$CO$_2$-alk; -NH-CO-alk; -CO-NH-alk; -alk-B; -CH(OH)-alk-B; -alk-Asp-Arg-NH$_2$; -CH(OH)-alk-Asp-Arg-NH$_2$;

 ; ; or ;

R^{4A}, which provides a spacer arm, the relative rigidity of which is an aspect of the design, terminating in an acid group which mimics the tyrosine hydroxy groups of angiotensin II in its "receptor bound" conformation includes the following:

where Z is a bond, -NHCO-, -O-, -OCH$_2$-, or -CH$_2$-;
X is -CO$_2$H, -alk-CO$_2$H, -PO$_3$H, -alk-PO$_3$H, -PO$_4$H$_2$, -alk-PO$_4$H$_2$, -SH, -alk-SH, -SO$_3$H, -alk-SO$_3$H, -SO$_4$H$_2$, -alk-SO$_4$H$_2$, F$_3$C-CO-NH-, F$_3$C-SO$_2$-NH-,
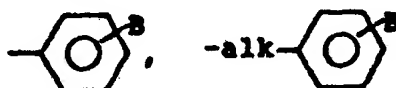
5   or yet another acid group
or a pharmaceutically acceptable salt thereof; and
Y is -H, -halide, -NO$_2$, -O-alk, -alk, -CF$_3$, or -CN; and
        R^{4B}, which optionally provides a spacer arm
terminating in a mimic of the N-terminus or N-terminal
10   dipeptide of angiotensin, includes the following:
-H, -alk-B, -alk-Asp-Arg-NH$_2$, alk-O-alk-B,
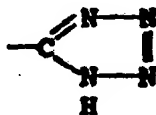alk-O-alk-Asp-Arg-NH$_2$,

preferably with the proviso that when R^{4B} is H, then:
(a) if the ring is imidazole α and/or γ is other
15   N, (b) if the ring is other than imidazole either α is
C or β is N, (c) R^{1A} comprises a group containing an
amide, (d) R^2 comprises a group containing A, or (e)
R^3 comprises a group containing B or -Asp-Arg-NH$_2$;
        alk = an alkyl group having from 1 to 10
20   carbon atoms, a cycloalkyl group having 3-6 carbon
atoms, an alkenyl group having 2-10 carbon atoms, or an
alkynyl group having 2-10 carbon atoms;
        halide = -F, -Cl, -Br, or -I;
        A = an acid group or its pharmaceutical salt
25   and includes but is not limited to -CO$_2$H, -CO$_2$R*,
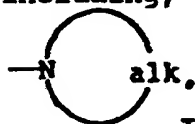-CO$_2$alk, -SO$_3$H, -SO$_4$H$_2$, -PO$_3$H, -PO$_4$H$_2$, F$_3$CCONH-, F$_3$CSO$_2$NH-,
    -alk-SH, or

SUBSTITUTE SHEET

-12-

wherein R⁺ is a lipophilic st r prodrug form such as
$-CH_2CO_2C(CH_3)_3$ and the like;

B = a basic group or its pharmaceutical salt
including, but not limited to $-NH_2$, -NHalk, $-N(alk)_2$,

$-N \overset{\frown}{\underset{\smile}{}} alk$.

In a particularly preferred product aspect of
the present invention, the above five-membered ring is
imidazole.

Another product aspect of the present
invention is directed to a compound of the formula:
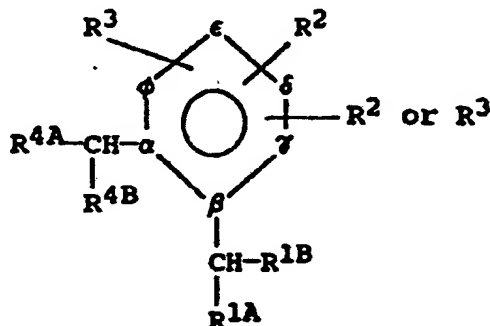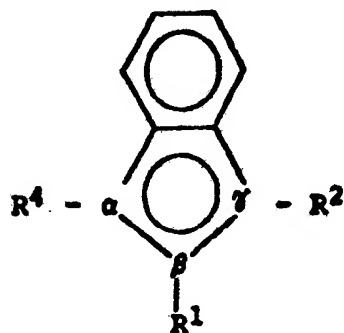

wherein α, β, γ, δ, ε, and φ are C, N, O or S
with the provisos that (a) the ring contains at least
one C atom and one N atom, and (b) attachment of R
groups is to C or N, and preferably further with the
provisos that (c) at least one ring N atom remains
unsubstituted, and (d) the pKa of the ring is ≤ 7 when
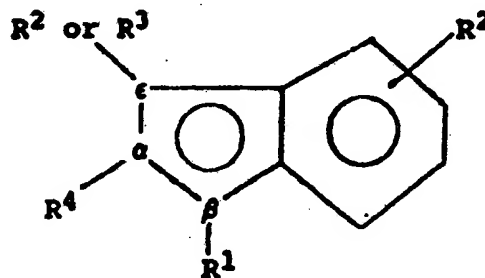all attendant groups have been taken into account;

-13-

and wherein R^{1A}, R^{1B}, R^2, R^3, R^{4A}, Z, X, and Y, R^{4B}, alk, halide, A, and B are as defined previously.

Another product aspect of the present invention is directed to a compound of the formula:

$R^4 - \alpha \quad \gamma - R^2$

β

R^1

Configuration I

R^2 or R^3 ⎯ ⎯ R^2

ϵ

α

$R^4 \quad \beta$

R^1

Configuration II

R^2

ϵ

α

$R^{4A} \quad \beta$

R^5
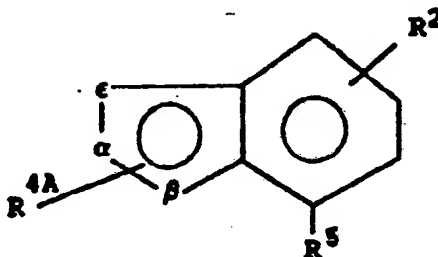
Configuration III

5   wherein α, β, and γ are C or N, with the proviso that only one N atom is substituted. R^1 is $-CH(R^{1A})(R^{1B})$ and R^4 is $-CH(R^{4A})(R^{4B})$. $R^5 = R^1$. Substituents R^{1A}, R^{1B}, R^2, R^3, R^{4A} and R^{4B} are as defined above, except that for these compounds when R^{1B} is H then (a) R^{1A} comprises a
10   group containing an amide, or (b) R^1 is on an N or (c) R^4 is on a C.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates a molecular model (three-dimensional spatial mod 1) for [Sar^1]Angiot nsin
15   II dev l ped by the methods of th present invention.

SUBSTITUTE SHEET

-14-

FIGURE 2A illustrates a two-dimensional representation of the Angiotensin II antagonist, Sarmesin, i.e., [Sar^{1}Tyr(Me^4)]Angiotensin II.

FIGURE 2B illustrates a two-dimensional representation of the Angiotensin II.

FIGURE 2C illustrates a two-dimensional representation of the Angiotensin II antagonist, Sarilesin, i.e., [Sar^{1}Ile^8]Angiotensin II.

FIGURE 3A illustrates a two-dimensional representation of one example of an N-benzyl-imidazole compound and FIGURE 3B illustrates a two-dimensional representation of one example of an N-benzamidobenzyl-imidazole, both compounds are in a class of compounds which are Angiotensin II antagonists.

FIGURES 4A illustrates a molecular model (e.g., a three-dimensional spatial model) for Angiotensin II developed by the methods of the present invention whereas FIGURE 4B illustrates a charge distribution map for Angiotensin II obtained obtained by overlaying the relative charges found in Angiotensin II onto the model illustrated in FIGURE 4A.

FIGURE 5A illstrates a two-dimensional structural formula of the imidazole portion of Angiotensin II whereas FIGURE 5B provides an overlay of the common portions of the compounds illustrated in FIGURES 3A and 3B and depicted by solid lines onto the imidazole portion of Angiotensin II illustrated in FIGURE 5A and depicted by dashed lines.

-15-

FIGURE 6 is a stereo photograph of a model of
Angiotensin II produced by the methods of this
invention.

FIGURE 7A is a stereo photograph of the
5   receptor bound form of Angiotensin II. FIGURE 7B is a
stereo photograph of the overlay of the compound
illustrated in FIGURE 7C over the receptor bound form
of Angiotensin II set forth in FIGURE 7A.

In the molecular models 2cm = 1 Å. One
10   skilled in the art will recognize that when the models
of Figures 6 and 7 are constructed from Minit Molecular
Models, Chocranes, Oxford, U.K., the color models will
provide even easier perception of the configurations
than the black and white stereo photographs herein
15   provide. (Additionally, it will be recognized by one
skilled in the art that in FIGURES 1, 4, 6 and 7, an
apparent error appears in that the valine and aspartic
amino acids are inadvertently depicted in the D rather
than their correct L configuration.)

20   DETAILED DESCRIPTION OF THE INVENTION
Although investigations on the conformation
of naturally occurring biologically active ligands such
as Angiotensin-II have heretofore been carried out,
such investigations generally did not take into account
25   the presence of a charge-transfer interaction in the
ligand which is required for receptor activation, and
therefore it has not heretofore been possible to
readily model ligands as well as mimetics of such
ligands to a meaningful conformation. However, by the
30   methods of th pres nt invention which do tak into
account charg -transfer int ractions, it is now
possible to model bi logically active ligands having

-16-

active site(s) which employ charge-transfer
interactions to a meaningful conformation. Moreover,
it is also possible to use the methods of the present
invention to model mimetics of such ligands. However,
5   prior to discussing this invention in detail, the
following terms will first be defined.

"Charge-transfer interaction" -- is an
electrostatic interaction involving a phenol residue in
which an anionic charge is transferred from a charged
10   group to an uncharged group. In one embodiment, the
phenol residue is initially uncharged, i.e., phenol,
and as a result of the charge-transfer interaction,
this residue accepts an anionic charge from another
charged group; thus in this embodiment the phenol
15   residue becomes a phenolate residue. In another
embodiment, the phenol residue is initially charged,
i.e., phenolate, and as a result of the charge-transfer
interaction, this residue transfers its anionic charge
to an originally uncharged group; thus in this process,
20   the phenolate residue becomes a phenol residue.

Any phenol residue found in a biologically
active ligand can be employed in the charge-transfer
interaction. Suitable phenol residues include those
found in the amino acid tyrosine and derivatives
25   thereof, in steroids having a phenol group such as
estradiol [estra-1,3,5(10)-triene-3,17,diol] and
derivatives thereof, in catecholamines such as
norepinephrine and derivatives thereof, in naphthol
containing ligands and the like. The above list is not
30   meant to be an exhaustive representation of naturally
occurring components employing phenol residues but
rather is presented for the purpose of illustrating

-17-

that such phenol residues can be found in many
different biologically active ligands.

"Active sites based on charge-transfer
interactions" -- refers to activation site(s) in a
5   biologically active ligand (for activating a
biologically active receptor) which is (are) based on
an electrostatic interaction involving a phenol residue
in which an anionic charge is transferred from a
charged residue to an uncharged residue. Accordingly,
10   in such interactions at least one of the residues is
either a phenol residue or a phenolate residue. In
such ligands, activation of the receptor by the ligand
cannot occur without the charge-transfer interaction.
Charge-transfer interactions have heretofore been
15   suggested for ligands such as Angiotensin II. See, for
instance, Moore et al., Bioscience Reports, 5,
pp. 407-416 (1985), which proposed that tranfer of a
negative charge from the C-terminal carboxylate residue
through the imidazole residue of the histidine amino
20   acid to the tyrosine side chain results in the
formation of a phenolate species which upon interaction
with the receptor activates the Angiotensin II
receptor. Such charge-transfer interactions allow the
ligand to modify its electrostatic character into a
25   form which allows activation of the receptor.

The charge-transfer interaction need not be
an electrostatic interaction confined solely to the
ligand but also could involve a transfer of charge from
either a residue on the ligand to a residue on the
30   receptor, or from a residue on the receptor to a
residue on the ligand, said transfer being a necessary
pr condition t activation of the receptor by the

-18-

ligand. For example, th formation of the tyrosinate
species on the ligand can be the result of the transfer
of an anionic charge from an anionic residue on the
receptor. Upon formation of the tyrosinate species,
5   the ligand is then capable of activating the receptor.

The methods of the present invention employ
techniques which permit detection of charge-transfer
interactions in biologically active ligands or in
10   biologically active ligand/biologically active receptor
complexes. These techniques employ a fluorescence
analysis discussed below in a fluorescence compatible
environment.

"Ligand" -- any organic compound for which a
15   receptor naturally exists or can be prepared.

"Biologically active ligand" -- a molecule
which binds to a biologically active receptor molecule
and which directly or indirectly affects the activity
of the receptor molecule. Binding of such ligands to
20   the receptor (acceptor) molecule is accordingly a
necessary precondition for initiating, terminating,
altering or preventing the biological activity in the
receptor molecule. Any ligand which affects the
biological activity of the receptor molecule is said to
25   be a biologically active ligand. The biologically
active ligand can be a substrate, an agonist, an
antagonist, an activator, an inhibitor, etc. When a
ligand is able to bind to a specific receptor, the
ligand and receptor pair are said to be complementary.
30   Examples of biologically active ligands are well
documented in the art. Examples of important
bi logically active ligands includ , for example,

-19-

oxytocin (wherein the presently known complementary receptors are oxytocin receptor and oxytocin-neurophysin), vasopressin (wherein the presently known complementary receptors are the V_1 receptor, the V_2

5   receptor, and vasopressin neurophysin), Angiotensin II (where the presently known complementary receptor is known as the Angiotensin II receptor), and the like.

The biologically active ligand can be peptidic or non-peptidic in nature. Such ligands can

10   be indigenous to the organism where the biologically active receptor is found. When the ligand is one which is naturally occurring in that organism, then that ligand is referred to as a naturally occurring biologically active ligand. On the other hand, the

15   biologically active ligands can be synthetic molecules which are complementary to the biologically active receptor and which affect the biological activity of the receptor. Thus any molecule which is complementary to a biologically active receptor and which affects the

20   biological activity of the receptor, is a biologically active ligand.

When binding of the biologically active ligand to the biologically active receptor and the activation of the active site results in an alteration

25   of the biological activity of the receptor, e.g., initiates, increases, decreases or terminates the biological activity of the receptor, the ligand is said to directly affect the activity of the receptor. On the other hand, a biologically active ligand indirectly

30   affects the activity of the biologically active rec ptor when the binding of the ligand to the rec ptor results in an inability t activate the rec ptor

-20-

(because the ligand possess a compromised charge-
transfer interaction--as in the case of a antagonist).

Activation of the active site of the
naturally occurring biologically active ligand/receptor
5   complex is generally accomplished by some sort of
chemical interaction within the ligand or between the
ligand and the receptor. As noted above, when the
chemical interaction involves the transfer of charge
from one residue to another wherein one of the residues
10   is either a phenol or a phenolate residue, the
interaction is termed a charge-transfer interaction.
Such charge-transfer interactions are believed to
result in the alteration of the structure of the ligand
or the ligand/receptor complex which then activates the
15   receptor. Because such charge-transfer interactions
can now be detected by the techniques employed in the
present invention, it is now possible to incorporate
such interactions into the model created for the
naturally occurring biologically active ligand and to
20   create agonists and antagonists to the complementary
receptor.

Preferably, when analyzed by nuclear magnetic
resonance spectroscopy employing the nuclear Overhauser
effect (as defined below), the ligand should have a
25   molecular weight of less than about 15,000 daltons, and
more preferably, less than about 10,000 daltons, even
more preferably, less than about 5,000 daltons and most
preferably, less than about 3,000 daltons. However, in
the fluorescence analysis of this invention, any
30   molecular weight biologically active ligand can be
employed.

-21-

"Angiotensin II" -- refers to the biologically active ligand which is an octapeptide represented by the amino acid sequence of

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

5   wherein each of the above abbreviations are art recognized abbreviations for amino acids.

"Oxytocin" -- refers to the biologically active ligand which is a nonapeptide represented by the amino acid sequence of

10   Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$

wherein each of the above abbreviations are art recognized abbreviations for amino acids.

"Vasopressin" (arginine vasopressin) -- refers to the biologically active ligand which is a

15   nonapeptide represented by the amino acid sequence of

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$

wherein each of the above abbreviations are art recognized abbreviations for amino acids.

"Receptor" -- a molecule which binds the

20   ligand.

"Biologically active receptor" -- a molecule, having a specific binding site for its complementary ligand, and can include classical hormone receptors, binding and/or transport proteins, enzymes, antibodies

25   and the like. One embodiment of a biologically active receptor includes membrane bound proteins which control certain cellular processes and which themselves are regulat d by the binding (or lack f binding) f its

-22-

complementary naturally occurring biologically active
ligand. Because such membrane bound biologically
active receptors are bound to membrane, it is believed
that the conformation of the biologically active ligand
5   necessary to activate such receptors are lipid induced.
See, for instance, Sargent et al., Proc. Natl. Acad.
Sci. (USA), 83(16), pp. 5774-5778 (1986) and Surewicz
et al., J. Amer. Chem. Soc., 110, pp. 4412-4414 (1988).
On the other hand, there are other biologically active
10   receptors which are not membrane bound. In such cases,
such receptors may not require a lipid induced
conformation of the biologically active ligand and, in
fact, may require an aqueous induced conformation of
the complementary biologically active ligand in order
15   to activate such receptors.

          Examples of biologically active receptors
have been well documented in the art. Specific
examples include insulin receptor (wherein the
complementary ligand is insulin), the V1 receptor
20   (wherein the complementary ligand is vasopressin), the
V2 receptor (wherein the complementary ligand is
vasopressin), oxytocin-neurophysin (where the
complementary receptor is oxytocin), the Angiotensin II
receptor (wherein the complementary ligand is
25   Angiotensin II), and the like.

          "Agonist" -- A biologically active ligand
which binds to its complementary biologically active
receptor and activates the latter either to cause a
biological response in the receptor or to enhance pre-
30   existing biological activity of the receptor. The
agonist can be the naturally occurring biologically
active ligand or it can be a synthetic molecule which

-23-

can also activate the receptor. For example, it is known in the art that Angiotensin II acts as an agonist for its complementary receptor, the Angiotensin II receptor. Other examples of agonists for the

5   Angiotensin II receptor include [Sar^1]Angiotensin II and the like. Examples of agonists for other receptors include norepinephrine (for its complementary receptor the alpha or beta adrenergic receptors). A common characteristic of all agonists in this invention is

10 that the charge-transfer interaction in the agonist which is necessary to activate the biologically active receptor is not compromised. That is to say that the charge-transfer interaction is operable in the agonist.

15   "Antagonist" -- A biologically active ligand which binds to its complementary biologically active receptor and either prevents the activation of the latter or deactivates the latter so as to either prevent or diminish the biological activity of the

20 receptor. For example, it is known in the art that the non-peptides 2-n-butyl-1-[4-carboxybenzyl]-4-chloroimidazole-5-acetic acid) and (methyl 2-n-butyl-1-[4-(2-carboxybenz-amido)benzyl]-4-chloroimidazole-5-acetate, sodium salt act as antagonists of the

25 Angiotensin II receptor. See Hypertension, 13, No. 5, May 1989. Other examples of art recognized antagonists to the Angiotensin II receptor include the peptides sarmesin, and the like. Examples of art recognized antagonists to other biologically active receptors

30 include propranolol for the ß-adrenergic receptor, cimetidine for the Histamine-H$_2$ receptor and the like. A common characteristic of all antagonists in this invention is that the charge-transfer interaction in the antagonist which is nec ssary to activate the

-24-

biologically activ receptor is compromis d. That is to say that the charge-transfer interaction in the antagonist is impaired and accordingly, the antagonist cannot activate the complementary receptor. For example, one method of impairing the charge-transfer interaction is to modify the hydroxyl group from the phenol moiety by, for example, methylating, (e.g., forming the $-\phi-O-CH_3$ group). Another method of impairing the charge-transfer interaction is to remove the hydroxyl group from the phenol moiety, e.g., changing phenol to phenyl.

For example and as noted above, Moore et al., Bioscience Reports, 5, pp. 407-416 (1985), proposed the presence of a charge-transfer interaction in Angiotensin II among the C-terminal carboxylate residue, the histidine amino acid and the tyrosine amino acid. In view of this charge-transfer interaction, two classes of antagonists to Angiotensin II are recognized; both of which have an impaired charge-transfer interaction. The first class involves antagonists in which the tyrosine hydroxyl group is modified or deleted and in which the N-terminal amino acid has been modified (e.g., [Sar^{1}Tyr(Me)^4]Angiotensin II, Sarmesin). The other class of antagonists to Angiotensin II involves antagonists in which the C-terminus is modified, with or without concomitant modification of other parts of the molecule (e.g., [Sar^{1}Ile^8]Angiotensin II, Sarilesin].

Thus, while an antagonist is a biologically active ligand, it is not a biologically active ligand having an active site based on a charg -transfer

-25-

interaction because, by d finition, this charge-transfer interaction has been impaired.

"Mimetics" -- refers to agonists and antagonists to a biologically active receptor but which have a different structural formula (primary structure) than the naturally occurring biologically active ligand for said receptor. That is to say that mimetics are non-naturally occurring biologically active ligands.

"Tertiary structure of a biologically active ligand" -- refers to the art recognized term which describes the three-dimensional _in vivo_ organization of the individual atoms of such ligands including the charge distribution map so generated. The tertiary structure of a biologically active ligand (often termed its "conformation") reflects non-covalent interactions between/among atoms as well as covalent bonding between atoms. Non-covalent interactions include both electrostatic and non-electrostatic interactions such as ionic bonds, hydrogen bonding, Van der Waal forces, etc. Because the extent and nature of such non-covalent interactions are dependent on the polarity of the solvent in which they are measured, the tertiary structure (conformation) of such ligands will change when taken from its _in vivo_ micro-environment and placed into an environment of different polarity.

"Fluorescence compatible environment" -- is an environment where long lifetime fluorescence (LLF - defined hereinbelow) can be detected. In this regard, it is noted that certain solvents such as dimethylsulfoxide (DMSO) and water do not permit detection of LLF, presumably because of such factors as

solvent induced fluorescenc qu nching, solvent
interference with intramolecular hydrogen bond
formation. On the other hand, the use of aqueous
solutions of micelles and lipid bilayers as well as the
5   use of solvents having a dielectric constant of about
40 or less allows for detection of LLF. Preferably,
solvents having a dielectric constant of less than 40
are employed as the fluorescence compatible
environment. Even more preferably, the dielectric
10   constant fluorescence compatible environments is from
about 2 to about 40. Suitable solvents having a
dielectric constant of about 40 or less include, for
instance, propylene glycol, isopropanol,
trifluoroethanol and the like. Lastly, the solvent so
15   selected should itself not possess fluorescence in the
region where the LLF is being detected.

"Receptor-simulating environment" -- refers
to an environment created to simulate the polarity of
the in vivo micro-environment in the immediate vicinity
20   of a biologically active receptor. As noted above, if
a biologically active ligand is placed into an
environment of different polarity from its in vivo
micro-environment, its tertiary structure will change
but not its structural formula, i.e., the covalent
25   bonds will not change. The addition of a biologically
active ligand into a receptor-simulating environment
allows the ligand to substantially conform to the
tertiary structure it would possess if placed in the
micro-environment of its complementary biologically
30   active receptor. For example and as noted above, for
membrane bound biologically active receptors, it is
believed that the conformation of a biologically active
ligand responsible for activating the recept r is lipid

-27-

induced. Accordingly, for such receptors, the
receptor-simulating environment will be less polar than
aqueous environments and solvents having a dielectric
constant of about 50 or less have been found to provide
5   a receptor-simulating environment for such membrane
bound receptors. Suitable solvents having a dielectric
constant of about 50 or less include dimethylsulfoxide
(DMSO), trifluoroethanol, isopropanol, propylene
glycol, and the like. For non-membrane bound
10   receptors, a solvent having a dielectric constant of
about that of water or less will provide a receptor-
simulating environment.

"Three-dimensional spatial model of a
biologically active ligand" -- refers to the tertiary
15   structure of such a biologically active ligand created
from the analytical techniques herein described. The
creation of such three-dimensional spatial models is
sometimes referred to herein as "modelling".

Because the NMR techniques which are employed
20   to create the three-dimensional spatial model employ a
receptor simulating environment, the model created will
substantially conform to the biologically active
ligand's tertiary structure. However, because the
polarity of the solvent simulating enviroment will not
25   be exactly the same as the _in vivo_ micro-environment,
the three-dimensional model will possess minor
variations from the tertiary structure. Provided that
a receptor simulating environment is employed, the
resulting variations will be minor in nature and the
30   three-dimensional spatial model will provide meaningful
information concerning th _in vivo_ t rtiary structur
of the bi logically active ligand.

-28-

"NMR spectroscopy using the Nuclear
Overhauser effect" -- refers to the nuclear magnetic
resonance methodology which permits insights into the
three-dimensional spatial organization of the ligand's
5   atoms.   Suitable NMR methodologies include proton [^{1}H]
NMR, ^{19}F NMR, ^{13}C NMR, and the like.   Preferably, proton
NMR is employed.

The first step of this methodology employs
Correlated Spectroscopy ("COSY") which is a two-
10   dimensional NMR spectrum yielding information on
through-bond coupling patterns within a molecule.   COSY
methodology permits the assignment of individual proton
resonances within the spectrum to particular protons in
the ligand.   This information is then used to identify
15   NOE correlations.   Such COSY methodology is well known
in the art and is described by Cheatham, Journal of
Chemical Education, 66, pp. 111-117 (1989).   In some
cases, COSY methodology can be supplemented by ROESY
and 1-D NOE methodologies in the assignment of
20   individual proton resonances within the spectrum to
particular protons in the ligand.

Once the two-dimensional assignments have
been made via the COSY methodology, the next step is to
conduct nuclear magnetic resonance (NMR) employing the
25   nuclear Overhauser effect methodology [such as one-
dimensional NOE enhancement, two-dimensional NOESY and
two-dimensional ROESY (rotating frame nuclear
Overhauser effect spectroscopy)] on the ligand.   NMR
employing the nuclear Overhauser effect methodology is
30   used to describe a change in intensity of one NMR line
when another line is irradiated at the frequency of the
latter line.   The chang in intensity is due to

-29-

"through space" energy transfer from one atomic nucleus
to another. Thus, the nuclear Overhauser effect
provides information of nearest neighbor atomic nuclei
to the line that is saturated. Accordingly, the
5   accumulation of a sufficient number of the nuclear
Overhauser effects among neighboring atoms can be used
to determine the spatial characteristics for the entire
molecule.

The nuclear Overhauser effect is a well known
10   and art recognized NMR effect and is described by
Cheatham, Journal of Chemical Education, 66, pp. 111-
117 (1989). This reference describes the use of one-
dimensional NOE enhancement as well as the use of NOE
in 2-dimensional NMR (NOESY) as a tool to create three-
15   dimensional models. The ROESY method is also well
known and art recognized and is described by Bax and
Davis, J. of Magn. Reson., 63, pp. 207-213 (1985). The
use of ROESY is particularly suitable for intermediate
size molecules such as peptide hormones.

20   "Fluorescence analysis" -- refers to the
identification of a charge-transfer system in a
biologically active ligand by using a fluorescence
instrument capable of measuring fluorescence decay at
the level of a nanosecond, or shorter, time intervals.
25   Such equipment is known in the art and is commercially
available, for example, from Photochemical Research
Associates under the tradename System 3000.
Fluorescence decay due to an excited-state phenol (or
phenolate) species involved in the charge-transfer
30   interaction is determined in a fluorescence compatible
environm nt after excitation with light of a suitable
wav length. For exampl , if tyrosine is involv d in

-30-

the charge-transf r interaction s as to result in a
tyrosinate species, fluorescence decay due to excited-
state tyrosinate emitting at and around 350 nm is
determined after excitation with light of a suitable
5   wavelength, e.g., 275 nm. Other excited state species
(e.g., ligands with phenol containing groups other than
tyrosine) involved in the charge-transfer interaction
can also be determined by measuring their fluorescence
decay at a suitable wavelength after excitation at an
10   appropriate wavelength. The appropriate wavelengths of
absorption and emission can be readily determined by
the skilled artisan for any given phenol containing
ligand.

        The experimentally obtained fluorescence
15   decay, which is described as a sum of exponentials, is
deconvoluted, and the lifetime of the longest component
due to the phenolate species of interest is determined.
Methods for summing the exponentials to obtain the
fluorescence decay, deconvolution of the fluorscence
20   decay and determining the lifetime of the longest
component due to the phenolate species are known in the
art and exemplified in the examples set forth
hereinbelow.

        Long lifetime fluorescence ("LLF") -- is the
25   half-life of the longest living fluorescent component
emitting at or around the species' fluorescent maximum
and is employed to determine the existence of a stable
charge-transfer interaction occurring in the ligand.
In particular, in tyrosinate excited-state fluorescence
30   analysis in propylene glycol, LLFs greater than about
11 nanoseconds and preferably greater than about 12
nanoseconds are diagn stic that th tyrosinate moiety

-31-

or modified tyrosinate moiety is participating in a
stable charge-transfer interaction. Such diagnosis is
made on the basis that LLF's greater than about 11
nanoseconds for tyrosine or modified tyrosine
5   containing ligands in propylene glycol correlate to the
presence of at least some (i.e., ≥1% relative to
Angiotensin II) agonist activity for said ligands. On
the other hand, a LLF of 11 nanoseconds or less in
propylene glycol is indicative that the tyrosinate
10   species or modified tyrosinate species responsible for
the LLF is not sufficiently stable and does not
activate the receptor. Again, such diagnosis is made
on the basis that LLFs of about 11 nanoseconds or less
for tyrosine or modified tyrosine containing ligands
15   correlate to inactive or antagonist activity for said
ligands (agonist activity of less than 1% relative to
Angiotensin II). Similar correlations to determine
whether a species different from tyrosine in a ligand
is participating in the charge-transfer interaction can
20   be made based on the LLFs of this species or modified
species in a variety of ligands correlated to whether
the particular ligand is an agonist, is an antagonist
or is inactive.

Without being limited to any theory, it is
25   believed that the charge-transfer interaction imparts a
level of stability to the excited state of the species
(e.g., tyrosine), which permits a longer LLF for the
species. Accordingly, longer LLFs correlate to the
presence of a charge-transfer interaction which in turn
30   correlate to agonist activity.

Having d fined the terms used herein, the
inv ntion will now be described in detail.

-32-

As noted abov , the first step in the preparation of a three-dimensional spatial model of a biologically active ligand having one or more charge-transfer interactions is a fluorescence analysis of the biologically active ligand. That is to say that the ligand is analyzed using fluorescence techniques in order to determine the existance of a charge-transfer interaction. In the following description of this fluorescence technique, Angiotensin II will be employed as a representative ligand. However, it is understood that other biologically active ligands can be analyzed in the same manner as Angiotensin II by using the methods hereinbelow described for Angiotensin II.

Nanosecond time-resolved fluorescence decays of Angiotensin II and analogs thereof were measured by taking advantage of the characteristic fluorescent properties of the excited-state tyrosinate species (other phenolate species would also exhibit similar characteristic properties for their excited-state). In this regard, in order for fluorescence emission from tyrosinate (and other phenolate species) to occur, there must be proton transfer to/from the phenolic hydroxyl group from/to an appropriate acceptor group. Based on the pKa's of tyrosine in the ground state (10.4) and in the excited state (less than or equal to about 5.4), protolysis in the excited-state is more efficient.

In particular, nanosecond time-resolved fluorescence decays of Angiotensin II and analogs thereof were measured from the emission at 350 nm due to its excited-state. Long lifetime fluorescence (LLF) was determined for each of th se analogs in several

-33-

solvents of different polarity using N-acetyl-tyrosine-
amide as the reference standard. The results of this
analysis demonstrate that solvents such as water and
DMSO do not allow detection of long lifetime

5   fluorescence in these analogs; presumably because of
factors such as solvent induced fluorescence quenching,
solvent interference with intramolecular hydrogen bond
formation, etc. On the other hand, use of a
fluorescence compatible environment such as aqueous

10   lipid bilayer solutions, micelles in an aqueous
environments, and solvents having a dielectric constant
of about 40 or less permit the detection of long
lifetime fluorescence.

Without being limited to any theory, it is

15   believed that this detection of the long lifetime
fluorescence in a fluroescence compatible environment
is due to the fact that such environments either do not
quench the fluorescence generated by the tyrosinate
excited-state and/or do not interfere with

20   intramolecular hydrogen bonding in Angiotensin II.
Additionally, as noted above, that Angiotensin II
conformation (tertiary structure) which permits the
formation of a charge-transfer interaction will
stabilize the tyrosinate excited-state which in turn

25   results in very long lifetime fluorescence. Insofar as
the conformation of Angiotensin II is not stagnant but
in fact is dynamic (i.e., in a given environment at a
given temperature, Angiotensin II is constantly
changing conformation both _in vitro_ and _in vivo_), only

30   that conformation which permits formation of the
charge-transfer interaction responsible for receptor
activation will result in the formation of a very long
lifetime fluorescenc . Accordingly, the environm nt

-34-

used for determining the presence of a charge-transfer
interaction via such fluorescence analysis should be
selected to be compatible with the fluorescence
analysis and to allow for the presence of that
5   conformation which permits this interaction. Such
results are achieved with the fluorescence compatible
environment employed in this invention. Preferably,
the fluorescence compatible environment will maximize
the presence of that conformation of such a
10   biologically active ligand which actives the receptor;
but such is not necessary provided that the
fluorescence compatible environment permits the
presence of a sufficient amount of the conformation of
the biologically active ligand which activates the
15   receptor so that its LLF can be detected.

         In membrane bound receptors, recent evidence
from site-specific receptor mutation studies suggests
that small ligands, i.e., ligands having a molecular
weight of less than about 3,000 daltons, bind to a site
20   in one of the transmembrane domains of the receptor
protein and therefore may have a biologically active
conformation which is lipid-induced. In such cases, it
is believed (again without being limited to such a
theory) that use of solvents of intermediate polarity
25   or less (i.e., having a dielectric constant of about 50
or less), lipid bilayers and micelles provides a
receptor environment which simulates the micro-
environment which the ligand encounters in the vicinity
of such membrane bound receptors. Thus use of a
30   fluorescence compatible environment for biologically
active ligands complementary to such receptors provides
the additional advantage that such environments should

-35-

facilitate the maximization of the ligand's conformer
responsible for activating the receptor.

5

10

Table I below shows the average long lifetime
fluorescence values obtained from Angiotensin II and
related analogs in isopropanol as well as propane-1,2-
diol (propylene glycol). Table I also shows the
agonist activity of Angiotensin II as well as for the
listed analogs. [The data set forth in Table I below
was obtained in a manner similar to that set forth in
Examples 1 and 3 set forth hereinbelow].

-36-

TABLE I

		SOLVENT			
		PROPANE-1,2-DIOL		ISOPROPANOL		AGONIST
LIGAND	LLF[a]	% LLF	LLF[a]	% LLF	ACTIVITY[b]
A	20.8	19	15.5	79	100
B	13.1	46	13.1	10	27
C	18.8	11	9.3	11	7
D	14.9	13	0	--	4[c]
E	16.2	10	0	--	5[c]
F	9.2	6	11.6	3	0.2
G	6.6	35	0	--	less than 0.1
H	10.6	17	9.4	16	less than 0.1
I	10.2	8	8.5	12	less than 0.1
J	0	--	6.5	20	less than 0.1[d]
K	7.4	10	10.2	14	10

a = in nanoseconds
b = Agonist Activity was measured via a rat isolated uterus bioassay as described by Matsoukas et al., J. Med. Chem., 31, pp. 1418-1421 (1988). Results are reported relative to Angiotensin II wherein Angiotensin II = 100.
c = Potent receptor antagonist with residual agonist activity.
d = Potent receptor antagonist.

Ligand A = Angiotensin II
Ligand B = [Sar[1]His(3-Me)[6]]Angiotensin II
Ligand C = [Sar[1]Phe[6]]Angiotensin II
Ligand D = [Sar[1]Cha[8]]Angiotensin II
Ligand E = [Des[1]Cha[8]]Angiotensin II
Ligand F = [Sar[1]Phe-NH$_2$[8]]Angiotensin II
Ligand G = [Sar[1]Ala[6]]Angiotensin II
Ligand H = [Sar[1]His(1-Me)[6]]Angiotensin II
Ligand I = [Sar[1]D-Pro[7]]Angiotensin II
Ligand J = [Sar[1]Ile[8]]Angiotensin II (Sarilesin)
Ligand K = Angiotensin III

The preparation of Ligands B-K is well known in the art. See, for instance, Matsoukas et al., Journal of Med. Chem., 31, pp. 1418-1421 (1988).

Sar = sarcosine
Cha = cyclohexylalanine
Des = amino acid residue omitted

-37-

In Table I above, % LLF measures the percent
of conformer(s) present which give rise to LLF.

The above data demonstrate that strong
agonists, Ligands A and B, possess a long lifetime
5   fluorescence in isopropanol of greater than 13
nanoseconds as compared to Ligands possessing either
low agonist activity, antagonist activity or
inactivity, Ligands C-K. Likewise, in propylene
gylcol, Ligands (except Angiotensin III) possessing any
10   agonist activity, Ligands A-E, possess a long lifetime
fluorescence of greater than 11 nanoseconds, whereas
Ligands either possessing no activity or antagonist
activity without any residual agonist activity, Ligands
F-J, possess a long lifetime fluorescence of 11
15   nanoseconds or less. Accordingly, prolonged duration
of the long lifetime fluorescence correlates to agonist
activity which in turn indicates that tyrosine's phenol
residue is involved in the charge-transfer interaction
responsible for receptor activation.

20   Contrasted with the readily conducted method
of this invention which establishes that tyrosine is
involved in the active state of Angiotensin II via
fluorescence analysis, the prior art had previously
determined that the tyrosine hydroxyl group of
25   Angiotensin II played an important role in receptor
activation either by preparing Angiotensin II analogs
without tyrosine or by methylating the hydroxy group of
tyrosine. It is clear that the process of the present
invention is more facile and does not require the
30   synthesis of numerous analogs of Angiotensin II.
M reover, modification of Angiotensin II by removal of
amino acids etc., can in fact change th tertiary

-38-

structure of the analog r lative to Angiotensin II such
that meaningful conclusions may be difficult to reach.

Once a charge-transfer interaction has been
identified in the ligand via the fluorescence analysis
5   of this invention, the next step in the process of
preparing a three-dimensional spatial model of a
biologically active ligand having one or more charge-
transfer interactions is a determination of the
chemical groups involved in the charge-transfer
10   interaction. Such a determination can be conducted by
using art recognized structure activity relationships.
In this regard, these determinations are greatly
facilitated by the knowledge that a phenol/phenolate
species is involved in the charge-transfer interaction.
15   Accordingly, in those ligands having only one such
species (e.g., tyrosine) it is readily apparent that
such a species is involved in the charge-transfer
interaction.

In general, structure activity relationships
20   are conducted by creating analogs of the ligand of
interest by selectively replacing or modifying one of
the components of the ligand (e.g., in the case of a
peptide, an amino acid), and then determining the LLFs
of the analogs. Reduction in the LLF of an analog as
25   compared to the ligand is significant evidence that the
component originally found in the ligand and
subsequently replaced or modified in the analog plays a
role in the charge-transfer interaction. See Ligands F
and G in Table I which identify the histidine and C-
30   terminal carboxylate in the charge-transfer interaction
in Angiotensin II. Additionally, loss of agonist
activity in the analog provides corroborating evidence

-39-

that the component plays a role in the charge-transfer
interaction. In this regard, if the ligand contains
two or more phenol/phenolate species, determination
which of such species are involved in the charge-
5   transfer interaction can be made by creating analogs in
which one of the two or more phenolic groups has been
compromised, by for example, methylating the hydroxy
group. Analysis of the LLFs and biological activities
of such analogs will provide the required information
10   to determine which of the two or more phenolic groups
is involved in the charge-transfer interaction.

Once the groups involved in the charge-
transfer interaction have been identified, the next
step in the process of preparing a three-dimensional
15   spatial model of a biologically active ligand having
one or more charge-transfer interactions is to resolve
remaining aspects of the ligand's three-dimensional
spatial conformation by obtaining conformational
information relative to the active site from nuclear
20   magnetic resonance spectrscopy employing the nuclear
Overhauser effect.

As noted above, this step first involves the
use of COSY methodology which provides information on
through-bond coupling patterns within a molecule and
25   allows for the two-dimensional assignment of individual
protons in the ligand. The COSY methodology is
established in the art. After the two-dimensional
assignment of the individual protons via COSY
methodology, the ligand is then examined by conducting
30   nuclear magnetic resonance employing the nuclear
Overhauser effect m thodology. Suitable nuclear
Overhaus r effect methodologies include one-dimensional

-40-

NOE enhancement, two-dimensional NOESY and two-dimensional ROESY. All of these nuclear Overhauser effect methodologies are established in the art.

5   However, with regard to ligands having a molecular weight of between about 500 to 2000 daltons, the use of NOESY methodology often fails for such ligands, irrespective of the internuclear distances involved, because the tumbling rate for these solutes is close to that at which the maximum possible NOE

10   passes through zero. See Bax and Davis, J. Magn. Reson., 63, pp. 207-213 (1985). Consequently, sequential assignments and the observation of interproton distances revealing structures are impossible using NOESY for such ligands. However, such

15   ligands can be structurally analyzed using either one-dimensional NOE enhancement or ROESY methodologies.

       In further regard to nuclear magnetic resonance spectroscopy using the nuclear Overhauser effect, there is a practical limit on the molecular

20   weight of the ligand being analyzed. In particular, ligands having a molecular weight of about 15,000 daltons or greater impose to much complexity on current NOESY/ROESY methodologies to permit their use. However, in certain circumstances, one-dimensional NOE

25   methodologies could be used. Accordingly, in this invention, ligands being investigated by nuclear magnetic resonance spectroscopy employing the nuclear Overhauser effect preferably have a molecular weight of less than about 15,000 daltons and preferably have a

30   molecular weight of less than about 10,000 daltons.

-41-

The solvents used when conducting proton
nuclear magnetic resonance spectroscopy employing the
nuclear Overhauser effect are selected so as to provide
a receptor simulating environment. Thus, if the
5   biologically active receptor is a membrane bound
receptor, current hypotheses suggest the role of lipid-
induced peptide folding in peptide hormone-receptor
interactions. See Sargent et al., Proc. Natl. Acad.
Sci. (USA), 83(16), pp. 5774-5778 (1986); and Surewicz
10   et al., J. Amer. Chem. Soc., 110, 4412-4414 (1988).
Such lipid-induced peptides are generally believed to
have a molecular weight of less than about 3,000
daltons. Thus, in these circumstances, the use of
solvents having a dielectric constant of about 50 or
15   less is justified. Furthermore, such dielectic
constants allow for a more ordered peptide structure.

A particularly preferred solvent for use in
nuclear magnetic resonance spectroscopy employing the
nuclear Overhauser effect for ligands whose
20   complementary receptor is a membrane bound receptor is
dimethylsulfoxide (DMSO). In particular, DMSO is
preferred because it offers several advantages over
other possible solvents having a dielectric constant of
about 50 or less for the following reasons: 1) the
25   solvent allows for the buildup of NOEs to a level of
detectability which is not possible in solvents such as
deuterated water; 2) for the reasons noted above, the
bulk dielectric environment provided by DMSO is such
that it represents an environment not unlike that
30   encountered by such peptides at their receptors, and
which gives useful and practical information; 3) the
sp ctra ar charact riz d by sharp and well resolved
prot n signals which can b individually assign d using

-42-

COSY methodology and are often superior to spectra
obtained in solvents such as trifluoroethanol,
propylene glycol and isopropanol which give broader and
often overlapping signals; 4) DMSO is superior to
5   aqueous environments for charged molecules because
fewer conformations are usually sampled and
conformational averaging is altered in DMSO as compared
to water; and 5) the dielectric constant of DMSO (.45)
is sufficiently close to the maximum dielectric
10   constant employed in the fluorescence analysis so that
minimal conformational changes are expected in the two
enviroments.

When the receptor simulating environment is
aqueous in nature, the use of water or a solvent
15   mixture containing water is justified.

In any event, when the NMR methodologies
described hereinabove are proton [^{1}H] NMR
methodologies, deuterated solvents will be required,
i.e., d_6-DMSO, D_2O and the like.

20   Examples 4-6 hereinbelow set forth
biologically active ligands which have been analyzed by
nuclear magnetic resonance spectroscopy employing the
nuclear Overhauser effect. In this regard, Examples 1-
3 had already established that the tertiary structures
25   for naturally occurring biologically active ligands
(i.e., [Sar^1]Angiotensin II and oxytocin) employ a
charge-transfer interaction to activate the
biologically active receptor and which groups were
involved in the charge-transfer interaction.

-43-

Further in this regard and by using the methods of the present invention, molecular models of biologically active ligands have been developed. In particular, FIGURE 1 illustrates a molecular model of

5   [Sar']Angiotensin II. In FIGURE 1, the backbone of [Sar']Angiotensin II is maintained by two gamma turns maintained in part by hydrogen bonds between the Arg CO and Tyr NH and between His CO and Phe NH (not shown). [All of the molecular models depicted herein were

10   developed using Minit Molecular Models, Cochranes, Oxford, U.K. A person skilled in the art can readily reproduce such models.]

For comparison purposes, FIGURES 2A, 2B and 2C illustrate simplified two-dimensional structures

15   showing some conformational aspects of Sarmesin, Angiotensin II and Sarilesin, respectively. In reality, the aromatic rings lie above the peptide backbone. See FIGURES 1 and 4A.

FIGURE 4A illustrates a molecular model of

20   Angiotensin II determined in DMSO/D_2O by 2D-ROESY proton NMR in a manner similar to that of Example 4. The backbone of Angiotensin II is characterized by two gamma turns maintained in part by hydrogen bonds between the Arg CO and Tyr NH and between His CO and

25   Phe NH (not shown). FIGURE 6 illustrates three-dimensional stereo photographs of the model of Angiotensin II. In regard to the figures containing stereo photographs, it is noted that such photographs should be viewed by stereo glasses/viewer in order to

30   obtain th three-dimensional effect. Such st reo glasses/view rs ar commercially availabl ;

SUBSTITUTE SHEET

-44-

one source being Marivac, 1872 Garden St. Halifax, Nova
Scotia, B3M 3RL, Canada.

Once a three-dimensional spatial model for a
biologically active ligand has been developed using the
techniques of this invention, further refinement of
this model or development of even new models can be
accomplished using theoretical considerations. For
example, with knowledge of the three-dimensional model
for Angiotensin II depicted in FIGURE 4A and
illustrated in the stereo photographs of FIGURE 6,
it is possible by employing theoretical
considerations to create a three-dimensional model for
Angiotensin II bound to its receptor, the Angiotensin
II receptor. In particular, such theoretical
considerations generally relate to readily available
chemical pathways. For instance, because of the
charge-transfer interaction, the tyrosine hydroxyl
group in Angiotensin II has been converted to its
tyrosinate species. The tyrosinate species, which is a
strong nucleophile, can then be derivatized by the
receptor resulting in transient bonding between the
ligand and the receptor. Upon such bonding, tyrosine
moves away from the histidine side chain because the
histidine is no longer able to form a hydrogen bond
with the tyrosine hydroxyl group. Moreover, slight
repositioning of the histidine is also expected. Such
theoretical considerations have already been forwarded.
See, for instance, Moore et al., Int. J. Pept. Prot.
Res., 26, pp. 469-481, (1985). In view of the above, a
receptor bound three-dimensional spatial model of
Angiotensin II was developed which accounts for such
conformational changes which would occur if Angiotensin

SUBSTITUTE SHEET

-45-

II behaves in the suggested manner. A stereo
photograph of this model is depicted in FIGURE 7A.

         The validity of the resulting model can be
readily verified by overlaying known antagonists onto
5   the receptor bound model and ascertaining whether the
antagonists can conform to the model. That is to say
that if the model is correct, then the antagonists
should be able to adapt a conformation similar to the
model of the ligand so as to bind to the receptor and
10   thereby account for their antagonist behavior. In this
regard, FIGURES 3A and 3B illustrate examples of
compounds from a class (i.e., structurally related
compounds) of antagonists of Angiotensin II. This
class is generically known as either N-benzyl-imidazole
15   compounds ("BI") or N-benzamidobenzyl-imidazole
compounds ("BABI"). It is noted that in FIGURE 3B, the
acidic proton in BABI is present in a hydrogen bonded
form (depicted by the box around this proton together
with the dots to the amido carbonyl group) somewhat
20   analogous to the hydrogen bonded form of the tyrosinate
species of Angiotensin II. Many acidic groups can
exist in similar hydrogen-bonded stabilized forms in
BABI compounds, e.g., carboxylate (shown), sulfate,
trifluoro-methylsulfonamido, and the like. It is also
25   noted that for the BI class of compounds, the acidic
proton can occupy a similar position in space to the
acidic proton shown in BABI, but that the former is not
stabilized by hydrogen bonding.

         In FIGURE 5B, the common portion of these
30   antagonists hav b en overlayed onto the imidazol
portion f Angi tensin II depict d in FIGURE 5A which
additi nally shows the r lativ position of th

-46-

compon nts responsible for the charge-transfer
interaction in Angiotensin II (note--the imidazole
double bonds have been removed from FIGURE 5B for the
sake of clarity). In FIGURE 5B, the fact that the
5   hydroxyl group of the hydroxymethyl in both antagonists
is similarly located to the tyrosine hydroxy group in
Angiotensin II; the fact that the n-butyl side chain
of both antagonists mimics precisely the His C_β-His C_α-
His CO-Pro N chain of Angiotensin II; and the fact that
10   the chlorine atom in these antagonists can serve to
decrease the basicity of the imidazole nucleus of these
compounds, indicates that the antagonist can form a
conformation with similar electronic and three-
dimensional characteristics as the conformation of
15   Angiotensin II required for generation of the charge-
transfer interaction responsible for activating the
receptor. However, because these antagonists lack the
necessary functionality to generate a charge-transfer
interaction, they can not activate the receptor which
20   accordingly explains their antagonist properties.

Having generated a model for the tertiary
structure of a biologically active ligand, it is now
possible to design and synthesize mimetics to this
ligand. For example, it is now possible to design and
25   synthesize compounds which are sufficiently similar to
the model generated for the tertiary structure of the
biologically active ligand so as to be complementary to
the ligand's receptor. In this regard, antagonists are
created when the compound so designed and synthesized
30   has a compromised charge-transfer interaction whereas
agonists are created when the compound so designed and
synthesized has an operable charge-transfer
interaction, i.e., the charge-transf r interacti n is

-47-

not impaired. With knowledge of the model generated
for the tertiary structure of a biologically active
ligand, the design and synthesis of agonists and/or
antagonists to the ligand's complementary receptor is
5   well within the ability of the skilled artisan.

        The present invention also offers a
particular advantage in the design and synthesis of new
mimetics optionally based on the structure of known
mimetics coupled with knowledge of the model generated
10   for the tertiary structure of a biologically active
ligand. This particular advantage is especially
applicable to designing new mimetics of Angiotensin II,
which may or may not be based on the structure of known
mimetics.

15   In particular, structure-activity
relationships show that the binding affinity between
Angiotensin II and its complementary receptor derives
largely from Coulombic [ionic] forces originating from
complementary charges between Angiotensin II and its
20   receptor. The ionic charges on Angiotensin II are
illustrated in FIGURE 4B which is based on the model
for Angiotensin II depicted in FIGURE 4A. In FIGURE
4B, N⁻ denotes tyrosinate which relocates upon
interaction with the receptor (this is shown in FIGURE
25   7A which is a stereo photograph of receptor bound
Angiotensin II). On the other hand, the N-benzyl-
imidazole (BI) and N-benzamidobenzyl-imidazole (BABI)
class of known antagonists to Angiotensin II [Wong et
al., Hypertension, 13, pp. 489 et seq., (1989)] are
30   devoid f many of the charges which cause Angiotensin
II to bind tightly to its rec ptor. Ov rlay of th
imidaz le group f the BI and BABI compounds d pict d

SUBSTITUTE SHEET.

-48-

in FIGURES 3A and 3B onto the imidazole group of the
model for Angiotensin II depicted in FIGURE 5A is
illustrated in FIGURE 5B. The three-dimensional
organization of the chemical groups of BI and BABI

5   compounds is such that these compounds can mimic
1) Angiotensin II, 2) Sarmesin, or 3) Sarilesin.
(For example, when the imidazole-based hydroxyl group
of BABI compounds is methylated, the resulting
oxymethyl group occupies a similar position in space to

10   the oxymethyl group of Sarmesin.) Overlay of a
specific BABI compound (depicted in FIGURE 7C) onto the
receptor bound model of Angiotensin II (depicted in
FIGURE 7A) is illustrated in the stereo photograph of
FIGURE 7B. As can be seen from FIGURE 7B, because the

15   BABI com-pound has a similar spatial arrangement to the
Tyr-Val-His sequence of Angiotensin II (as well as to
Sarilesin), the BABI compounds can mimic this portion
of the model of Angiotensin II (and Sarilesin) so as to
be complementary to the Angiotensin II receptor.

20   Further in this regard and without being limited to any
theory, it is believed that the receptor may
transiently acylate, or the like, the tyrosine hydroxyl
group of receptor bound Angiotensin II and alter the
location of the tyrosine side-chain relative to its

25   position in the "charge-transfer interaction" form of
Angiotensin II. Moreover, it is further believed that
the acidic portion of BABI compounds which is
stabilized in a predisposed or "preactivated" form
by a hydrogen bonding interaction with the carbonyl

30   oxygen of the amido group (See FIGURE 3B), occupies a
position in space which is similar to that of the
tyrosine hydroxyl group in the receptor bound model
for Angiotensin II depicted in FIGURE 7A.
It is still further b lieved

SUBSTITUTE SHEET

-49-

that a bond, similar to that formed between the
"preactivated" tyrosinate group of Angiotensin II and a
receptor-based acceptor group, will also be formed
between the "preactivated" acid group of BABI compounds
5   and the receptor. In contrast to Angiotensin II, it is
also believed that for the case of BABI compounds, this
will not result in receptor activation because of the
different conformational constraints and the nature of
the ligand-receptor bond. Thus, for example, if the
10   receptor acylates the tyrosine OH group of Angiotensin
II, the adduct formed between Angiotensin II and its
receptor will involve an ester bond, whereas that for
the BABI compound shown in FIGURE 3B will involve an
anhydride linkage or for the BABI compound shown in
15   FIGURE 7C will involve an amide linkage. This
discloses the fact that BABI compounds are Angiotensin
II receptor antagonists because they can act as
transition state inhibitors or suicide substrates for
the Angiotensin II receptor. In contrast to BABI
20   compounds, BI compounds are not likely to act by this
mechanism because the acidic proton is not stabilized
by hydrogen bonding, and is therefore not preactivated.

       In any event, it is seen that the BI/BABI
class of compounds commonly possess an imidazole ring
25   which can be modified to enhance the potency of these
compounds.

       This information, in conjunction with the
charge distribution map depicted in FIGURE 4B, allows
for the design and synthesis of new antagonists to the
30   Angiotensin II receptor based on incorporating
additional charges at th appropriat location into BI
and BABI compounds so as to increase the binding

-50-

affinity of these antagonists to the Angiotensin II
receptor and accordingly increase their potency.   In
view of the above, derivatives of the BI and BABI
compounds having one or more such charges can be
5   prepared as follows [from FIGURE 4B, it can be seen
that all charges (except the tyrosinate charge)
including the imidazole ring lie in the same
approximate plane:

          1. All distances are given relative to the
10   center of planar imidazole ring (either of the His
amino acid in Angiotensin II or of the imidazole ring
of the BI/BABI compounds).

          2. The placement of the charges is defined by
a line drawn through the center point of the imidazole
15   ring which bisects the N_3-C_4 bond of imidazole ring of
histidine as shown in Formula I as follows:
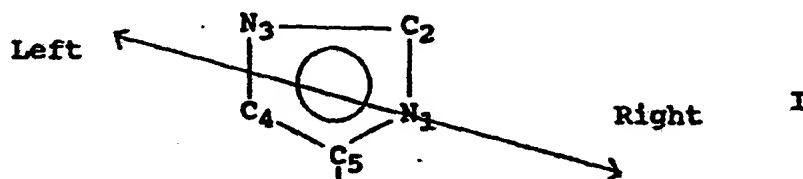
          wherein the subscripts 1-5 correspond to
accepted numbering of a histidine imidazole ring. [For
20   analogs in which the His ring is rotated through 180°,
e.g., His(3-methyl) analogs, the bisected bond becomes
the N_1-C_2 bond.]

          3. One or more of the following charges can
be placed onto the imidazole ring:

25   i) Direction: Left
                   Charge: Cationic
                   Distance from center of imidazole
                   ring: 7 ± 1.5 Angstroms
                   (Corresponds to N-terminus cationic
30   charge);

-51-

    ii) Direction: Right
       Charge: Anionic
       Distance from center of imidazole
       ring: 2.5 ± 0.5 Angstroms
       (Corresponds to C-terminus anionic
       carboxylate charge);

    iii) Direction: Left
       Charge: Anionic
       Distance from center of imidazole
       ring: 10 ± 2 Angstroms
       (Corresponds to aspartic acid anionic
       charge); and

    iv) Direction: Left
       Charge: Cationic
       Distance from center of imidazole
       ring: 12 ± 2.5 Angstroms
       (Corresponds to arginine cation)

    4. In the above, the orientation of the imidazole rings of Angiotensin II and the BI/BABI compounds is as shown in Formula II as follows:

ANG II His   BI/BABI   II
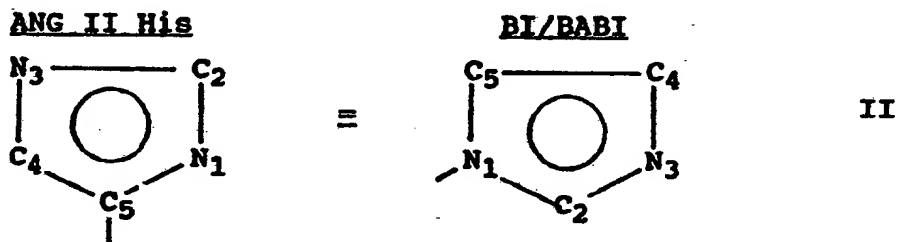
Thus C_4 in Angiotensin II is equivalent to N_1 in the BI/BABI class of compounds.

-52-

Examples of side chains which can be added to
BI/BABI compounds include for instance compounds of the
following Formula III:
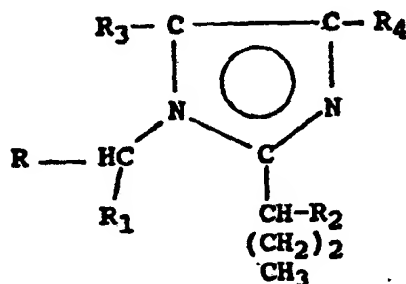
$$R_3 - C \text{————} C - R_4$$

(structure of Formula III)

III

wherein R is selected from the group consisting of
a) phenyl para substituted with a substituent selected
from the group consisting of carboxyl or a
pharmaceutically acceptable salt thereof, sulfate, and
trifluoromethylsulfonamido, and b) -NHC(O)R$_5$ wherein R$_5$
is phenyl ortho substituted with a substituent selected
from the group consisting of carboxyl or a
pharmaceutically acceptable salt thereof, sulfate, and
trifluoromethylsulfonamido, R$_1$ is either hydrogen or
hereinafter defined, R$_2$ is either hydrogen or as
hereinafter defined, R$_3$ is either hydroxymethyl,
-CH$_2$OCH$_3$, -CH$_2$C(O)OCH$_3$, -C(O)OCH$_3$, or as hereinafter
defined, and R$_4$ is either fluorine, chlorine or as
hereinafter defined.

In view of the above, a compound mimicking
the N-terminal cationic charge can be prepared by
attaching a suitable amino group at the appropriate
location on Formula III. Such a group could be placed
at the appropriate distance from the center of the
imidazole nucleus by employing an alkyl amino
substituent (or anoth r suitable cati nic group such as
a guanidino group, and th like) wherein the number of

methylene groups employed in the chain linking the
amino group to the BI/BABI compound is selected so as
to provide a positive charge at 7 ± 1.5 Angstroms left
from the center of the imidazole ring. For example,
placement at RR_3 of a $-(CH_2)_4-NH_2$ group will provide
such a charge (the amino group will protonate in the _in
vivo_ environment to form a $-NH_3^+$ group). Likewise, if
hydroxyl functionality is to be maintained at R_3, then
R_3 will be the group $-CHOH-(CH_2)_3-NH_2$. Alternatively,
the positive charge at 7 ± 1.5 Angstroms can be
obtained by placement at R_1 of a $-(CH_2)_3NH_2$ group. In
still another alternative, R_1 or R_3 can be
$-(CH_2)_n-Asp-Arg-NH_2$ wherein n is 3 for R_1 and 4 for R_3
which provides for the 3 charged groups found in the N-
terminal dipeptide of Angiotensin II. In regard to the
above, only one of R_1 and R_3 should be substituted at
any one time with a cationic group.

A C-terminal anionic mimetic can also be
prepared by placing a negative charge at right 2.5 ±
0.5 Angstroms to the center of the imidazole ring. For
example, placement at R_4 of a $-(CH_2)_3C(O)OH$ group will
provide the necessary negative charge at right 2.5 ±
0.5 Angstroms (the carboxyl group will deprotonate in
vivo to provide a carboxylate group, i.e., $-C(O)O^-$·
Alternatively, placement at R_2 of a $-(CH_2)_3C(O)OH$ will
provide the necessary negative charge at right 2.5 ±
0.5 Angstroms. In regard to the above, only one of R_2
and R_4 should be substituted at any one time with an
anionic group.

Similar considerations regarding the
attachm nt of charges can be appli d to th imid zol
group f th His amino acid so as to arrive at mim tics

-54-

to the Angiotensin II receptor. This is particularly
true because as indicated above, all of the ionic
charges in receptor bound Angiotensin II (except the
tyrosinate anion) are in approximately the same plane

5   and moreover, in approximately a straight line.
Moreover, the imidazole ring is planar and lies in the
same approximate plane as the ionic charges.
Accordingly, the substituents set forth above for the
imidazole group of BI/BABI compounds could be placed at

10   their equivalent points on the imidazole of the His
amino acid.

Similar groups can be designed for the
aspartic acid anionic charge and for the arginine
cationic charge.

15   The compounds depicted above can be readily
prepared by the skilled artisan using art recognized
techniques. Such compounds and their pharmaceutically
acceptable salts are useful as Angiotensin II
antagonists. Accordingly, such compounds can be used

20   to control hypertension and/or congestive heart failure
in a mammal in need of such treatment. Additionally,
the compounds of this invention are contemplated as
being useful in other cardiovascular and related
diseases such as stroke, myocardial infarction and the

25   like. When used to control hypertension and/or
congestive heart failure, the compound is normally
administered to such a mammal either orally or
parenterally. When so administered, the compound is
generally formulated in a pharmaceutically acceptable

30   diluent and at a dosage sufficient to control
hypertension and/or congestive heart failure in the
mammal so treated. The specific dos levels for such

-55-

uses can be readily determined by the skilled artisan.
Accordingly, the present invention contemplates a
method for controlling hypertension in a mammal in need
of such treatment which comprises either administering
orally or parenterally a pharmaceutical composition of
a compound depicted above in an amount sufficient to
control hypertension. Additionally, the present
invention also contemplates a method for treating
congestive heart failure in a mammal in need of such
treatment which comprises either administering orally
or parenterally a pharmaceutical composition of a
compound depicted above in an amount sufficient to
control said heart failure. The methods of controlling
hypertension are implemented using pharmaceutical
compositions comprising a pharmaceutically acceptable
carrier and an amount of a compound depicted above
effective to control hypertension in a mammal in need
of such treatment. The methods of controlling
congestive heart are implemented using pharmaceutical
compositions comprising a pharmaceutically acceptable
carrier and an amount of a compound depicted above
effective to control said heart failure.

The present invention will be described in
further detail with reference to the following
examples. However, it should be understood that the
present invention is by no means restricted by these
specific examples.

EXAMPLES

A.   Examples 1 and 3 below are directed to
the identification by fluorescence analysis of the
presenc r absenc of a tyrosinat charge-transfer
interaction in biologically active ligands. The

-56-

fluorescence analysis was measured on a nanosecond, or
shorter, time intervals. Fluorescence decay due to
excited-state tyrosinate emitting at and around 350 nm
was determined after excitation with light of a

5   suitable wavelength, e.g., 275 nm. The experimentally
obtained fluorescence decay, which is described as a
sum of exponentials, was deconvoluted, and the lifetime
of the longest component due to tyrosinate was
determined. In solvents of intermediate polarity, such

10   as propylene glycol, isopropanol and the like, or in
membrane environments, a lifetime in excess of about 11
nanoseconds for the long lifetime fluorescence of the
excited-state tyrosinate is diagnostic of the existence
of a particularly stable tyrosinate charge transfer

15   interaction in the subject material.

Example 1 -- Fluorescence Properties of Angiotensin II
            Angiotensin II was obtained from Sigma
(acetate form) and from Peninsula Labs
(trifluoroacetate form) and was found to contain a

20   single peptide by reverse-phase HPLC. Analogs of
Angiotensin II were synthesized, purified and
bioassayed by method described by Matsoukas et al., J.
Med. Chem., 31, pp. 1418-1421 (1988). 1,2-propanediol
[Pr(OH)$_2$] was dried by refluxing over calcium oxide for

25   8 hours, collected by distillation and stored over a
molecular sieve. Water content was estimated by ^{1}H NMR
or by the Karl Fisher method. Isopropanol (PrOH) was
of HPLC grade (Caledon Laboratories Ltd) and was
expected to contain less than 1% water. Dimethyl

30   sulfoxide (DMSO) and trifluoroethanol (TFE) were used
without further treatment. Aqueous solvents were
prepared from distilled water which had been passed
through Fish r i n-exchange cartridges. N-acetyl-

-57-

tyrosine-amide (NAYA) was obtained from Sigma. Sodium
dodecylsulfate (SDS) was obtained from BDH biochemicals
(specially pure) and was used without further
treatment.

5   Fluorescence experiments were performed at
21°C and sample concentration of Angiotensin II used
for fluorescence analysis were typically between 0.25
and 1.0 mg/mL. The samples can be warmed to about 50°C
to facilitate dissolvement of the peptide. If desired,
10   the samples can then be filtered. Cuvettes were
cleaned with sulfochromic acid and were soaked in the
highly purified solvent of the experiment. Nanosecond
time-resolved fluorescence decays were measured at 21°C
using Photochemical Research Associates (PRA)
15   fluorescence lifetime instrumentation (System 3000).
This instrument utilizes the time correlated single
photon counting technique. A PRA 510 flash lamp was
utilized as the light source and was operated at
18.6 kHz, with 5.8 kV applied across a 4mm electrode
20   gap under -44 kPa of H_2. The excitation and emission
wavelengths were selected using Jobin Yvon monochroma-
tors with slits giving an 8nm bandpass. The lamp decay
profile was obtained by measuring the scattering of
light by a suspension of 2.02μm polyvinyltoluene latex
25   spheres in glycerol/water (1:1) with the excitation and
emission monochromators set at the emission wavelength
of the sample. In all experiments data were collected
until 2.5 x 10^5 photon counts were obtained.
Background counts were obtained for each solvent and
30   were subtracted from the sample data; the background
obtained during the time of the sample collection was
less than 7% of the counts at the tail end of the
sample d cay. The bs rv d decay data were

deconvoluted beginning from 5 channels before the
channel maximum to the channel which contained the
0.05% of the photon counts present in the channel of
maximum counts. The deconvolution method used was that
of iterative non-linear least squares. See Grinvald et
al., Anal. Biochem., 59, pp. 583-598 (1974). Acceptance
of a least squares fit at 95% confidence was evaluated
by the reduced chi-squared test, and the quality of fit
was evaluated from the residuals, the autocorrelation
function of the residuals, and the Durbin-Watson para-
meter. See Lampert et al., Anal. Chem., 55, pp. 68-73
(1983).

The experimentally obtained fluorescence
decay, $f(\lambda,t)$, is described as a sum of exponentials:

$$f(\lambda,t) = \Sigma \, \alpha_i(\lambda) \, \exp \, [-t/r_i(\lambda)] \qquad (1)$$

where $\alpha_i(\lambda)$ and $r_i(\lambda)$ are the preexponential weighting
factor and fluorescence lifetime of the ith component
for a given emission wavelength, respectively. The
fraction of the fluorescence intensity that arises from
each component is related by:

$$Int \; \%(\lambda) = \frac{\alpha_i(\lambda)r_i(\lambda)}{\Sigma\alpha_i(\lambda)r_i(\lambda)} \times 100 \qquad (2)$$

Normalized fluorescence decay curves for
angiotensin II were obtained in propylene glycol,
isopropanol and 0.1 M aqueous SDS. Triexponential fits
to the data (equations 1 and 2) gave two parameters:
the lifetime of the longest fluorescence component
(LLF), and the percentage of the intensity arising from
the longest decay component % LLF. The observed LLF
for angiotensin II in propylene glycol was
20.8 nanoseconds and the perc nt LLF was 19. F r

-59-

angiotensin II in isopropanol the LLF was
15.5 nanoseconds and the percent LLF was 79. In
trifluoroethanol, angiotensin II gave LLF equals
13.0 nanoseconds and percent LLF equals 19. In aqueous
5   SDS (SDS above the critical micelle concentration),
angiotensin II gave LLF equals 13.7 nanoseconds and
percent LLF equals 14.

        The finding that the addition of SDS above
its critical micelle concentration in water induces
10   tyrosinate fluorescence suggests that intramolecular
hydrogen bond formation of the tyrosine hydroxyl in
Angiotensin II could occur in the presence of a cell
membrane but not in its absence. Both the stability of
the tyrosinate species (LLF) and the percent conformer
15   providing for the tyrosinate species (% LLF conformer)
were significantly increased when the SDS micelles were
formed in the presence of Angiotensin II (LLF = 13.7)
compared to when preformed SDS micelles were added to a
solution containing Angiotensin II (LLF = 7.2). The
20   former represents a situation where Angiotensin II
becomes trapped within the hydrophobic interior of the
micelles, whereas the latter represents binding of the
positively charged Angiotensin II to the negatively
charged exterior surface of the micelle. Differences
25   in tyrosinate fluorescence in these embodiments
indicate that the tyrosinate species is stabilized even
in an extremely non-polar (hydrophobic) environment,
i.e., environments having a dielectric constant of
about 2. Accordingly, such non-polar environments are
30   receptor simulating environments.

        Analogs of angiotensin II with agonist
activities less than 1% of th ag nist activity f

-60-

angiotensin II in the rat uterus assay had LLF less
than 11 nanoseconds in propylene glycol, and analogs
with about 10% or less agonist activity had LLFs less
than 12 nanoseconds in isopropanol. For example,
5   [Sar^{1}Ile^8]Angiotensin II gave LLF equals 0 in propylene
glycol and LLF equals 6.5 nsec in isopropanol.

          Changing the concentration of the sample did
not affect the parameters obtained, therefore
dimerization or multiple aggregates can be ruled out as
10   possible conformations responsible for the LLF
component.

          The above data demonstrates that the
existence of a charge-transfer interaction involving
the tyrosinate residue in a specific ligand can readily
15   be determined by evaluating the LLF of the tyrosine
moiety in the ligand.

          Example 2 -- Structure Activity Relationship
                       For Angiotensin II

20   Structure activity relationships were
conducted on Angiotensin II by preparing the analogs
[Sar^{1}Ala^6]Angiotensin II and [Sar^{1}Phe-NH$_2$]Angio-
tensin II. The LLFs, % LLF and Agonist Activity for
these analogs are set forth in Table I above. The
25   absence of LLFs greater than about 12 nanoseconds in
these analogs implicates both the imidazole and C-
terminal carboxylate of Angiotensin II in the charge-
transfer interaction. Additionally, the lack of
significant agonist activity in the analogs
30   corroborates this finding.

-61-

Example 3 -- Fluorescence Properties and Structural Activity Properties of Oxytocin

Using the fluorescence technique described
above, oxytocin had an LLF equal to 18.5 nanoseconds in
5   propylene gylcol. On the other hand, an analog of
oxytocin, [Ala^5], had an LLF of 7.6 nanoseconds in
propylene gylcol. Accordingly, this establishes that
the asparagine occupying position 5 in oxytocin is
involved in the charge-transfer interaction.
10   Additionally, this analog possessed no agonist
activity, which corroborates this finding.

NMR SPECTROSCOPY

Conformational analysis of ligands is
achieved by 2D COSY coupled with 1D NMR, 1D NOE
15   enhancement or 2D NOE (ROESY) methods, using a receptor
simulating solvent. If the receptor simulating solvent
does not contain exchangable deuterium groups, then
small amounts of D_2O can optionally be added to the
DMSO for exchange purposes. NOE or ROE effects
20   observed as a result of intramolecular through-space
relaxations are recorded; interresidue NOEs illustrate
the proximity of neighboring groups and thereby provide
valuable conformational information. This information
is used to construct a molecular model of the ligand.
25   The procedure is facilitated if the presence of a
tyrosinate-forming interaction has already been
established by fluorescence spectroscopy.

Example 4 -- 2D-ROESY PROTON NMR STUDY OF [SAR^1]ANGIOTENSIN II

30   [Sar^1]Angiotensin II was synthesized by the
solid phase techniqu and purified to homogeneity by
reversed-phase HPLC using methods described by

-62-

Matsoukas et al., J. Med. Chem. 31(7), pp. 1418-1421
(1988). The synthetic peptide gave the required amino
acid analysis and appeared as a single product in two
thin layer chromatography (TLC) systems.

5   [Sar¹]Angiotensin II had 180% of the bioactivity of
Angiotensin II in the rat uterus assay which is also
described by Matsoukas et al., supra. Since HPLC
afforded the trifluoroacetate salt of the peptide,
[Sar¹]Angiotensin II was neutralized by passage through

10   a column (1.5 x 3 cm) of carboxymethylcellulose
(Whatman CM23) cation exchange resin. The peptide
(10mg) was first applied to the column in 0.01 M
ammonium acetate at pH 5 (5 ml) and then eluted with
0.5 M ammonium acetate at pH 8 (10 ml). The effluent

15   obtained at pH 8 was lyophilized thrice and 5 mg of the
product was dissolved in 0.5 ml of DMSO-d_6 and two
drops of D_2O were added. Argon was bubbled through the
sample for 5 minutes before the NMR tube was sealed.

       NMR experiments were carried out using a

20   Bruker AM 400 MHz NMR spectrometer, which was modified
to perform spin-locking with an effective radio
frequency field of 5 KHz at ambient temperature
(297 ± 1°K). Data acquisition and data processing were
controlled by an Aspect 3000 computer equipped with an

25   array processor using Bruker 1987 DISNMR software. The
chemical shifts were reported relative to the
undeuterated fraction of the CH_3 group of DMSO-d_6 at
2.50 ppm with respect to TMS. One-dimensional spectra
were recorded with a sweep width of 6100 Hz, and 32 K

30   (zero filled to 64 K) data points. A total of 64 scans
were accumulated to obtain a good signal-to-noise
ratio. Methods used were as described by Otter et al.,
Biochemistry, 27(10), pp. 3560-3567, (1988), and Marion

-63-

et al., Biochem. Biophys. Res. Commun., 113(3), pp. 967-974 (1983). The parameters employed in the two-dimensional NMR techniques are summarized in Table II below:

TABLE II

Summary of Experimental Parameters used in the Two-Dimensional NMR Experiments[e,f]

Parameters	Unit	COSY	ROESY PH
Sweep Width in F_2 (H_2)	Hz	4000	3300
Sweep Width in F_1 (H_2)	Hz	2000	1650
Matrix size ($F_1 \times F_2$) before zero filling	--	512x1K	256x1K
Matrix size ($F_1 \times F_2$) after zero filling	--	1Kx8K	1Kx2K
Evolution time initial value (μs)	μs	3	1
increment (ms)	ms		78
No. of scans (dummay scans)		32	64
Acquistion time	s	0.32	0.20
Relaxation delay (HDO presaturation)	s	1.8	1.8
Other delays[g]	ms		200
Window functions for 2D FT (F_1/F_2)		5/5	5/5
Shifts of window functions in fractions of π (F_1/F_2)		4/8	3/4

e = all spectra were recorded at 297°K at 10mM concentration in DMSO-d_6 (+ 2 drops D_2O)

f = After the Fourier transformation, the phase, was optimized in both dimensions by an additional phase correction applied to the entire matrix. The ROESY spectrum was baseline corrected in F^1 and F_2 by means of a Bruker ABS baseline correction.

g = Spin locking time, at an average rf field of 5 KHz.

Both pulse sequences incorporated a decoupler presaturation int rval to suppr ss th water signal. The resulting 2D matrices were displayed and slight

-64-

phase adjustment in both dimensions were usually
necessary to obtain the best possible data
representation. Correlations were verified by
examining individual rows and columns of unsymmetrized
5   and symmetrized spectra.

         The ROESY experiment required a basic 90°
phase correction in t_1 before the phase fine tuning
could be done. A carrier frequency of 3.7 ppm, a spin-
locking time of 0.2 sec. and 30° flip angle for the
10   hard pulse spin locking train were selected. It should
be noted that this experiment often suffers from
spurious resonances due to magnetization transfer
between scalar coupled spins. Under the selected
experimental conditions, the resulting two-dimensional
15   spectrum was almost free of such peaks, which are
otherwise easily identified by their phase being the
same as the diagonal signals (real ROE cross-peaks have
opposite signs with respect to the diagonal peaks).
Some baseline distortions were present, especially
20   around intense peaks such as the residual solvent
signals and methyl groups. To diminish this problem,
spectra were treated in both dimensions with the Bruker
ABS baseline correction routine using a polynomial
fitting of fifth degree to the baseline. Since all the
25   recorded two-dimensional spectra suffer from
considerable t_1 noise and ridges, caution was exercised
to obtain reliable information from the spectra.
Because only coupling connectivities were of interest,
we found it useful to record the magnitude spectrum in
30   the case of the COSY experiment; the ridges along t_1
can then be reduced considerably by methods described
by Otter et al., supra.

-65-

All resonances of the peptide were assigned
to individual amino acids by combined information from
COSY and ROESY spectra as set forth in Example 5 below.
Our assignments for [Sar¹]Angiotensin II differ from
those reported previously for Angiotensin II [by Smeby
et al., Chemistry and Biochemistry of Amino Acids,
Peptides and Proteins, Ed. Weinstein, Dekker, New York,
pp. 117-162 (1976)] only with regard to the relative
positioning of the Phe C_α proton within the C_α group.
Symmetrized and unsymmetrized ROESY spectra were
examined to identify both intraresidue and interresidue
cross-peaks. Table III below shows all ROESY
interactions, both intraresidue and interresidue:

TABLE III

Proton:proton ROESY interactions identified for
[Sar¹]Angiotensin II in DMSO-d_6 + $D_2O^{h,i,j,k,l}$

	Sar	Arg(R)	Val(V)	Tyr(Y)	Ile(I)	His(H)	Pro(P)	Phe(F)
Intraresidue interactions	α:M	α:β	α:β	α:β	α:β	β:β'	α:β	α:β
		α:β'	β:γ	α:β'	β:γ	α:C₄	α:β'	β:β'
		β:γ	α:γ	β:β'	β:γ'		β:γ'	α:Fφ
		γ:δ		α:m	γ:δ		γ:δ	β:Fφ
		α:γ		β:m	α:γ		γ':δ'	β':Fφ
		α:δ		β':m	α:γ'		δ:δ'	
		β:δ		m:o	γ:γ'			
					α:M			
					β:M			
Interresidue interactions	M:Yo		VM:YM	Ym:VM		Hα:Pδ	Pδ:Hα	Fφ:Yo
				Yo:Fφ		Hα:Pδ'	Pδ':Hα	Fφ:Pγ
				Yo:SarM			Pγ:Fφ	

h = M stands for methyl group protons.
i = Meta (m) and ortho (o) refer to the hydroxyl group
    on tyrosine.
j = The signals for phenylalanine ring protons (ⱷ)
    overlap and were not individually assigned.
k = β', γ', and δ' r f r to the upfield geminal proton
    resonance.

-66-

1 = The amino acid abbreviations us d below are the
      conventional art recognized abbreviations.

Interresidue interactions are extremely important for
studying the conformation of [Sar^1]Angiotensin II in
5   DMSO. Thus the cross-peaks for the Tyr ortho:Phe ring
protons, together with the cross-peaks for Pro C$_\gamma$:Phe
ring protons and Pro C$_\delta$:His C$_\alpha$ protons, illustrate the
proximity of Tyr with both His and Phe and suggest that
the three aromatic rings in Angiotensin II are in close
10 proximity.

    Useful information concerning the rotational
freedom of the three aromatic side chains was obtained
by examining the intraresidue ROE connectivities
between C$_\alpha$ and C$_\beta$ protons. For Tyr the interaction of
15 the C$_\alpha$ proton with the C$_\beta$ proton (δ=2.80 ppm) was much
stronger than the interaction of the C$_\alpha$ proton with the
C$_{\beta'}$ proton (δ=2.62 ppm), indicating hindered rotation
of the Tyr side chain. For Phe only the C$_{\beta'}$ proton
(i.e., the proton at δ=2.85 ppm) interacts with the C$_\alpha$
20 proton (δ=4.10) again indicating the presence of a
preferred rotameter and possibly less motion for the
Phe side-chain than the Tyr side-chain. For His
neither of the C$_\beta$ protons (δ_1=2.86 ppm and δ_2=2.75 ppm)
appeared to interact with the C$_\alpha$ proton (δ=4.60 ppm).
25 By examining rows and columns only a very weak
interaction could be observed between His C$_\alpha$ and C$_\beta$
protons; this suggests that the C$_\alpha$-C$_\beta$ bond of His may
be essentially fixed in the _trans_ form in DMSO.

    The interaction of the His C$_\alpha$ proton with
30 both Pro C$_\delta$ protons not only illustrates that the His^6-
Pro^7 bond exists primarily in the _trans_ f rm, but also

-67-

defines the orientation of the His-Pro bond. A further
connectivity present in the ROESY spectrum involves a
strong interaction between a methyl group of Val or Ile
with the C_α proton of Ile, Val or Phe. Definitive
5   assignment of these cross-peaks could not be made
because of signal overlap, but probably represent an
intraresidue interaction of Ile and/or Val. Similarly,
an interresidue interaction between Tyr meta and a
methyl group of Ile or Val could not definitely be
10   assigned. A weak connectivity was also observed
between the Sar NCH_3 proton (δ=2.23 ppm) and a Tyr
ortho proton (δ=6.58 ppm). A connectivity between the
Tyr ortho and His C_4 ring protons was observed in rows
but not in columns.

15   For Arg, the C_α proton (δ=4.32 ppm) appeared
to interact with all side-chain methylene protons.
Thus, connectivities were observed between the C_α
proton and 1) the two C_β protons (1.65 and 1.35 ppm),
2) the two C_γ protons (1.48 and 1.42 ppm), and 3) a C_δ
20   proton at 3.02 ppm (another connectivity between the
Arg C_α proton and a proton at 3.45 ppm was tentatively
assigned to a Arg C_δ proton). Non-equivalence of the
three geminal proton pairs may indicate restricted
rotation for the Arg side-chain. ROEs between C_α and
25   C_δ protons may illustrate folding of the Arg side-
chain.

Interactions between the Tyr ortho:Phe ring
protons, together with connectivities between the Phe
ring:Pro C_γ protons and His C_α:Pro C_δ protons, suggest
30   that all three aromatic rings in Angiotensin II are in
cl se proximity and form a cluster in DMSO. No ROE
cross-p aks were observed between the His and Phe

-68-

residu s. A pr vious report d scribing shielding of
the His ring by the Phe ring in DMSO [see Matsoukas et
al., Biochem. Biophys. Res. Commun., 122(1), pp. 434-
438 (1984)] may reflect indirect effects, resulting
5   from clustering of the three rings. Alternatively, the
His and Phe rings may be separated by a distance which
allows for an electrostatic interaction but is beyond
the maximum range (<5 Angstroms) for an observable ROE.
Similar considerations may explain the absence of an
10   observable two-way ROE between the Tyr and His rings.

        The relative orientation of the rings to one
another in the cluster cannot be deduced without
further information. However, some helpful information
is supplied in the form of connectivities between non-
15   aromatic protons, as illustrated in Table III. In
particular, the observation of through space
interactions between the His C_α and the two Pro C_δ
protons defines the orientation of the His-Pro backbone
and demonstrates the predominace of the trans isomer in
20   DMSO. It can be deduced that the C-terminal Phe
residue must swing around through about 90° in order
that the Phe ring can interact with the central Tyr
ring (Table III). Modelling experiments illustrate
that the Phe residue is most likely to approach the
25   Tyr-Ile-His sequence from a γ turn originating at the
His-Pro bond.

        The ROESY spectrum shows strong intraresidue
$C_\beta/C_{\beta'}$ interactions in all three aromatic residues (Tyr,
His, and Phe), illustrating non-equivalence and
30   restricted rotation for these geminal protons.
Moreover, non-equivalent interresidue interactions
b tw n the C_α prot n and th two C_β protons were

-69-

observed for Tyr, only one C_α/C_β intraresidue
interaction was observed for Phe, and C_α/C_β
interactions for His were very weak. These finding
suggest that the $C_\alpha-C_\beta$ bonds of all three aromatic
5   side-chains are unable to freely rotate. For His the
$C_\alpha-C_\beta$ bond protons appear to be locked in the <u>trans</u>
position, whereas for Tyr and Phe the $C_\alpha-C_\beta$ bond
protons may be fixed (on the NMR time scale) between
the <u>gauche</u> and <u>trans</u> orientatins. Restricted rotation
10   for the Phe ring may originate from interaction with
the His side chain, the C-terminal carboxylate and/or
an interaction with the Pro ring. The latter
interaction is evidenced in the ROESY spectrum by
cross-peak connectivities between Phe ring and Pro C_γ
15   and $C_{\gamma'}$ protons. These interresidue interactions were
non-equivalent; the interaction of the Phe ring
proton(s) with the lower field Pro C_γ proton at $\delta=1.70$
ppm, appeared to be considerably stronger than the
interaction with the upfield Pro C_γ proton at $\delta=1.50$
20   ppm. This indicates that the Phe and Pro rings are
close but that the Phe ring probably approaches the Pro
ring in a non-parallel manner. These findings are in
agreement with previous proposals which have suggested
functional roles for the three aromatic side-chains,
25   and steric/spatial roles for Ile and Val. See Moore,
Pharmacol. Ther., <u>33(2-3)</u>, pp. 349-381 (1987). More
specifically, it has been suggested that motion of the
His and Phe side-chains would be inhibited by
interaction with the C-terminal carboxylate and each
30   other, whereas the Tyr side-chain would be constrained
by H-bonding with His. Such considerations could
explain why the $C_\alpha-C_\beta$ bond of His exists predominantly
in the energetically less favorable eclipsed
conf rmation, since th energy gain d from the charg -

-70-

transer interaction could overcome the energy loss due
to an eclipsed His C_α-C_β rotomer.

According to the overall structural features
suggested by the present ROESY experiment, certain
previously proposed interactions [see Moore et al.,
Biosci. Rep., 5(5), pp. 407-416, (1985)] are
permissable. Thus, modelling experiments illustrate
that the Tyr hydroxyl could hydrogen bond to an
imidazole ring nitrogen of the His residue and that the
C-terminal carboxylate could also interact with the His
ring. These interactions appear to be possible in DMSO
without introducing undue constraint in the molecule.
Proximity of the C-terminal carboxylate to one of the
His ring nitrogens would serve to increase the
polarization of the His ring dipole, thereby increasing
the basicity of the other His ring nitrogen and the
strength of its interaction with the Tyr OH. This
interaction may result in the production of a
tyrosinate species which activates the receptor.

The present investigations have shown an
interresidue interaction of the Sar[1] residue with the
Tyr[4] ring. [Sar[1]]Angiotensin was selected in part for
this study so that information on interactions of the
N-terminal could be readily observed. In this regard,
1D NMR spectra conducted for Angiotensin II and
[Sar[1]]Angiotensin II in DMSO have shown that the
aromatic region of these spectra are identical. The
importance of the Sar[1] residue in contributing to the
antagonist activity in Sarmesin is well documented and
previous studies have shown that the N-CH_3 of Sar[1] is
subjected to a shielding influence in a number of
angiotensin analogu s in DMSO. See Moore et al.,

-71-

Biosci. R p., 5(5), pp. 407-416, (1985). The results
of this ROESY example support previous suggestions
[Matsoukas et al., Peptides 1986, Ed. Theodoropoulos et
al., Berlin, New York, pp. 335-339 (1987)] that the Sar

5   NCH_3 interacts with the Tyr ring. In particular,
examination of the rows and columns containing the
Sar NCH_3 of the symmetrized and unsymmetrized ROESY
spectrum indicated a weak connectivity with a Tyr ortho
proton. Thus the source of the shielding effect on the

10   NCH_3 could be the Tyr ring, and the Sar NCH_3 group may
exist just at the limiting range for observing an ROE.

        Summarizing the above ROESY results, cross-
peaks between aromatic rings provided evidence that the
three aromatic rings of [Sar']Angiotensin II cluster

15   together. Cross-peaks between the His C_α proton with
both Pro C_δ protons illustrated that the His^6-Pro^7 bond
exists primarily in the trans form. Cross-peaks
between the Sar NCH_3 proton and a Pro ortho proton
illustrated proximity of the N-terminus of the peptide

20   with the Tyr ring. An observed cross-peak between the
Phe ring protons and the Pro C_γ protons illustrated
that the Phe ring is close to the Pro ring [as well as
the His ring, previously noted].

        The information obtained from the 2-D ROESY

25   experiment together with the fluorescence data showing
the presence of a tyrosinate charge transfer system,
enables the construction of a molecular model for
[Sar'] angiotensin II. This model is shown in
FIGURE 1.

30   Likewise, by following the procedur s s t
forth above and because similar cross-peaks have been

-72-

observed for angiotensin II, a molecular model of Angiotensin II has been constructed and is set forth in FIGURE 4A. Stereo photographs of this model are illustrated in FIGURE 6.

5   The model for Angiotensin II shown in FIGURE 4A differs from previously reported conformations. In particular, the N-terminus and the Phe ring have been repositioned in FIGURE 4 in order to accomodate the presently observed proximity of the Tyr ring with both 10   the N-terminus and the Phe ring. Repositioning of the Phe ring is also compatible with ab initio calculations of ring pairing interactions which have suggested a perpendicular-plate interaction for the His and Phe rings. Fowler et al., Biochem. Biophys. Res. Commun., 15   153(3), pp. 1296-1300 (1988).

Example 5 -- 1D-NOE Enhancement For Sarmesin

Sarmesin and [Des¹]Sarmesin were synthesized by in a similar solid phase technique described in Example 4 above. Purification by reversed-phase HPLC 20   afforded the trifluoroacetate salt of the peptides which were neutralized by passage through a carboxymethylcellulose column as described as well in Example 4 above.

NMR experiments were carried out using a 25   Bruker 400 MHz NMR spectrometer. 5 mg of peptide was dissolved in 0.5 ml of DMSO-d_6 and two drops of D_2O were added. Argon was bubbled through the sample for 5 min. before the NMR tube was sealed. Data acquisition and data processing were controlled by an Asp ct 30   3000 computer equipp d with an array processor using 1987 DISNMR software. Th chemical shifts wer

SUBSTITUTE SHEET

-73-

reported relative to the undeuterated fraction of the
methyl group of DMSO-d_6 at 2.50 ppm with respect to
TMS. One-dimensional spectra were recorded with a
sweep width of 4500 Hz, and 32 K (zero filled to 64 K)
5   data points. A total of 64 scans were accumulated to
obtain a good signal-to-noise ratio. The methods used
were similar to those reported by Otter et al., J. Am.
Chem. Soc., 109, pp. 6995-7001 (1987). The COSY (two-
dimensional correlated spectroscopy) experiments
10   provided contour plots which were symmetrized with
respect to the diagonal. The nonselective longitudinal
^{1}H relaxation times were determined in DMSO-d_6+D_2O (2
drops) using a 180-τ-90° plus sequence and are
presented in Table IV below.

15   TABLE IV
          Proton T_1 Relaxation Times (in seconds) for Sarmesin

     A.   Aromatic Side Chains

               _____His_____   ____Tyr(Me)____

20   C_2H 0.332   meta 1.180
     C_4H 0.481   ortho 1.505
                                          CH_3 0.994

     B.   Backbone protons

     __His__   _Tyr(Me)_   __Ile__   __Val__   Phe(Arg,Pro)

25   H_α 0.436 H_α 0.962 H_α 0.726 H_α 0.853 H_α 0.896

     C.   Other

                    _____Sar_____

               H_α 0.559 CH_3 0.805

-74-

Several τ values ranging between 0.01 and 10s were employed. Relaxation delays of up to 10s were used for T_1 measurements.

5   One-dimensional NOE enhancement measurements were carried out in the difference mode using multiple irradiation. Each of the selected lines was irradiated 50 times for 100 ms (total irradiation time 5.0s). Other irradiation times (0.2, 0.5, 1 and 3s) were also employed in some experiments to monitor the NOE build-

10   up. The multiple irradiation procedure allows a very low decoupler power setting (typically 10 dB lower than for a standard NOE experiment) so that it is possible to avoid partial saturation of resonances in close proximity. A total of 1000 scans for each line was

15   required, and total relaxation time was 2s. Under the experimental conditions used for the NOE experiments (low power, different τ preirradiation times, saturation of control areas), spin diffusion and partial saturation were visibly minimized for the

20   interactions under discussion. NOE enhancements were determined as the point increase in signal size per proton after saturation of a functionally distinct proton. Table V below sets forth the resulting NOE enhancements:

-75-

TABLE V

NOE enhancements for Sarmesin and [Des¹]Sarmesin[m]

Peptide	Proton(s) sat.	Enhancement	% proton	Rat.
A	Tyr(Me) C_α	Tyr(Me) $C_{\beta\beta'}$	8.7	C
A	Phe C_α [+Val, Ile]	Phe $C_{\beta\beta'}$	2.4	C
A	His C_α	His $C_{\beta\beta'}$	8.2	C
A	His C_α	Pro C_δ	11.3	D
A	His C_α	Pro $C_{\delta'}$	8.6	D
A	Arg C_α	Arg $C_{\beta\beta'}$	2.5	C
A	Arg C_α	Arg $C_{\gamma\gamma'}$	1.5	C
A	Arg C_α	Arg $C_{\delta\delta'}$	1.1	C
A	Sar C_α [+Pro C_δ]	Sar CH_3	1.2	C
A	Sar CH_3	Sar C_α	0.9	C
A	Sar CH_3	His C_β	1.1	D
A	Pro C_δ [+Sar C_α]	His C_α	5.0	D
A	Pro C_δ [+Sar C_α]	Pro $C_{\delta'}$	9.0	C
B	Tyr(Me) CH_3	His C_2	0.4	D
B	Tyr(Me) CH_3	His C_4	0.5	D
B	Tyr(Me) CH_3	Tyr(Me) ortho	6.7	C
B	Tyr(Me) CH_3	Tyr(Me) meta	3.2	E
B	Tyr(Me) meta [+Phe]	Tyr(Me) ortho	18	C
B	His C_2	Tyr(Me) meta	2.1	D
B	His C_2	Tyr(Me) ortho	1.6	D

m = in $DMSO\text{-}d_6$

A = Sarmesin
B = [Des¹]Sarmesin
C = intraresidue NOE
D = interresidue NOE
E = spin diffusion

Sarmesin and [Des¹] Sarmesin were subjected
to two-dimensional correlated spectroscopy (COSY) and
nuclear Overhauser enhancement (NOE) experiments,
suitable for resonance assignment and distance
information. It was possible to assign peptide
resonances to individual amino acids by combining
information from the COSY and NOE difference spectra.
The one-dimensional NMR sp ctra of Sarmesin and [Des¹]

-76-

Sarmesin in DMSO-d_6 showed a complex downfield region
with broad overlapping NH resonances indicating fast
exchange. To simplify the C_α proton and aromatic
regions and to study the intramolecular proton-proton
5   interactions between aromatic rings and interresidue
backbone protons, the NMR experiments were carried out
after the NH's were exchanged with D_2O.

The NOE experiments combined with the COSY
spectrum permit complete assignment of all backbone and
10   side-chain proton resonances. Saturation of the C_α
protons of His, Tyr(Me), and Phe (overlapped with the
C_α protons of Val and Ile), resulted in an enhancement
of the vicinal β protons revealing their pattern and
exact position in the crowded aliphatic region of the
15   reference spectrum. Thus, the NOE difference spectrum
which resulted after saturation of the His C_α proton at
δ=4.64 ppm, showed an AB quartet at δ=2.86 ppm
attributable to the two vicinal His C_β protons. This
interaction was measured to be 8.2%. Similarly, the
20   NOE difference spectra which resulted after saturation
of the Tyr(Me) C_α proton at δ=4.48 ppm and the Phe C_α
at δ=4.11 ppm, show an AB quartet for the vicinal
Tyr(Me) and Phe C_β protons at δ=2.77 ppm (8.7%) and
2.95 ppm (2.4%).

25   The NOE difference spectrum resulting after
saturation of the His C_α proton also shows two strong
resonances at δ=3.20 ppm (11.3%) and 3.49 ppm (8.6%)
due to enhancement of the two Pro C_δ protons. Both Pro
C_δ protons are almost equally affected, revealing close
30   proximity and equidistance of both of these protons
from the His C_α proton. This interaction, which also
has been bserv d for [Sar^1]Angiot nsin II in DMSO

(Example 4 above), confirms the presence of the <u>trans</u>
form of the His[6]-Pro[7] bond and permits insight into the
relative orientation of the His-Pro-Phe sequence. The
stereochemistry around the His-Pro bond in Angiotensin
5   II has been the subject of many investigations using
mostly ^{1}H and ^{13}C-NMR spectroscopy. The results of this
example using proton:proton NOE enhancement confirm
these findings and, furthermore, define the precise
orientation of the His-Pro bond.

10   Saturation of the C_α proton of the Arg and
Pro at $\delta=4.34$ ppm and at $\delta=4.21$ ppm, respectively,
revealed multiplet patterns for the respective vicinal
ß protons at $\delta=1.58$ ppm and $\delta=1.75$ ppm in the NOE
difference spectra. A resonance enhancement observed
15   at $\delta=3.02$ ppm (1.6%) upon saturation of the Arg C_α
proton was tentatively attributed to an interaction
with the Arg C_δ protons. This interaction, observed
also in [Sar^1]Angiotensin II during rotating frame
nuclear Overhauser effect spectroscopy studies (Example
20   4), illustrates proximity of the Arg C_α protons with
the Arg C_δ protons. Saturation of the Sar C_α protons
resonance at $\delta=3.2$ ppm resulted as expected in the
enchancement of the Sar CH_3 proton resonance at $\delta=2.26$
ppm (1.2%). Conversely, irradiation of Sar CH_3
25   enhanced the Sar C_α protons (0.9%). In addition,
enhancement of one of the His C_β protons was observed
after Sar NCH_3 saturation, implying proximity of the
Sar CH_3 protons to one of the His C_β protons. The
observed enhancement of the His C_α proton resonance at
30    $\delta=4.64$ ppm (5%) and the downfield Pro C_δ proton
resonance at $\delta=3.48$ ppm (9%) can be attributed to the
saturation of the upfield Pro C_δ proton resonance which

-78-

is overlapped with the Sar C_α proton resonance at
$\delta=3.20$ ppm.

To investigate the proposed proximity of the
Tyr and His rings, [Sar[1]]Angiotensin II and
5   [Des[1]]Angiotensin II (Angiotensin III) were methylated
at the Tyr hydroxyl so as to provide a suitable probe
(δ - 3.61 ppm) for investigating interaction between
the two aromatic rings. For these reasons, NOE
experiments were carried out by saturating the Tyr OCH_3
10   resonance and the His C_2 and C_4 proton resonances in
both analogs. Upon saturation of the Tyr OCH_3
resonance of [Des[1]]Sarmesin ([Tyr(Me)[4]]Angiotensin
III), weak enhancements of the His C_2 and C_4 proton
resonances at $\delta=7.47$ ppm (0.42%) and $\delta=6.85$ ppm
15   (0.52%), respectively, were observed. The weakness of
these interactions places the Tyr OCH_3 group at the
limit of the permissable distance (< 5 Angstroms) from
the His imidazole ring for the effect to be observed.
The cancellation of the Phe ring resonance at the
20   vicinity of the Tyr meta resonance suggests that the
observed effect may be real and not due to spin
diffusion. However, enhancement of the Tyr ortho and
meta proton resonances at $\delta=7.82$ ppm (3.21%) and $\delta=6.71$
ppm (8.86%) was observed upon saturation of the
25   Tyr OCH_3 protons in [Des[1]]Sarmesin. Whereas the former
is an expected Overhauser effect, the latter may result
from second-order magnetization transfer via the ortho
protons. The possibility cannot be ruled out that the
enhancements of His C_2 and C_4 in [Des[1]]Sarmesin result
30   from secondary NOEs relayed by Tyr ortho and/or meta
protons, although such considerations would still place
the Tyr and His rings in close proximity. Since
similar NOE enhancements of the His C_2 and C_4 prot ns

-79-

were not observed for Sarmesin, the presence of the N-
terminal Sar may subtlely alter the conformation of the
octapeptide and place the Tyr OCH_3 group just outside
the boundary for permissible and observable NOE
5   interactions with the His ring.

Saturation of the His C_2 proton resonance at
$\delta=7.47$ ppm in [Des^1]Sarmesin, resulted in enhancement
of the Tyr meta and ortho proton resonances at $\delta=7.08$
ppm (2.15%) and $\delta=6.71$ ppm (1.58%). However, upon
10   saturation of the Tyr meta proton resonance at $\delta=7.09$
ppm (overlapped with the Phe ring proton resonances),
no enhancement was observed for the His C_2 and C_4
protons at $\delta=7.47$ ppm and $\delta=6.85$ ppm. Only the Tyr
ortho proton at $\delta=6.71$ ppm (18%) in the aromatic region
15   under scrunity was enchanced. The latter saturation
serves as a control experiment to show the minimum
contribution of partial saturation to the enhancement
of the Tyr meta and ortho proton signals.

The NOE difference spectra for Sarmesin upon
20   saturation of the His C_α, Tyr(Me) C_α and Phe (Val, Pro)
C_α protons reveals intraresidue C_α/C_β proton
interactions in His, Tyr(Me), and Phe. The presence of
an interresidue His C_α/Pro C_δ proton:proton NOE defines
a predominantly trans conformation for the His-Pro
25   peptide bond of Sarmesin in DMSO. The trans isomer
also predominates in [Sar^1]Angiotensin II (Example 4),
illustrating that both agonist and antagonist maintain
this conformational property in DMSO. Furthermore, the
similar effects of saturation of the His C_α proton on
30   both Pro C_δ protons locates the His C_α proton midway
between the two Pro C_δ protons.

-80-

An interresidue proton:proton NOE was observed between the Arg C_α Arg C_δ protons after saturation of the former in Sarmesin. This could illustrate that the Arg side-chain does not exhibit complete freedom of motion in DMSO, or that it exhibits sufficient conformational freedom to sample many conformations. The role of this positively charged side-chain may be to contact a complementary anionic site on the receptor and assist in bringing about productive binding of Angiotensin II to its receptor. The same interaction has been observed in [Sar[1]]Angiotensin II (Example 4). This, together with considerations for the His-Pro bond in [Sar[1]]Angiotensin II and Sarmesin, illustrates similarities in certain aspects of the conformations of the agonist and the antagonist in DMSO.

Proximity of the Sar CH_3 protons with one of the His C_B protons in Sarmesin is evidenced by signal enhancement of the latter with saturation of the former. This suggests the presence of a bend in the N-terminal Sar-Arg-Val region of the molecule which allows proximity of Sar and His. Modelling experiments suggest that, for steric reasons, the N- and C- termini of the molecules probably approach the central domain from different sides, thereby creating an approximately S-shaped peptide backbone.

The NOE difference spectrum for [Des[1]]Sarmesin upon saturation of the Tyr(Me) methyl protons illustrates enhancement of the His C_2 and C_4 protons. Proximity of the Tyr(Me) methyl group and the His ring is in accord with the previously postulated hydrogen bonding interaction between the Tyr OH and the

His ring in Angiotensin II. Spin diffusion not withstanding, the similar NOE enhancements of both the His C_2 and C_4 protons suggests that the Tyr(Me) ring may have a perpendicular orientation relative to the

5   imidazole ring; this deduction is based on the expected planarity of the methoxy group with the Tyr ring. This is a potentially interesting observation since the relative orientations of the Tyr and His rings may be similarly maintained in the cluster of aromatic rings

10   in Angiotensin II. Even if enhancement of the His C_2 and C_4 resonances is due to secondary NOEs relayed by the Tyr ortho and/or meta protons, the data still establish the important fact that the Tyr and His rings are in close proximity.

15   Upon saturation of the His C_2 proton in [Des[1]]Sarmesin, NOE enhancement of the Tyr(Me) meta and ortho protons is observed. Due to overlap of the His C_4 and Tyr(Me) meta and ortho proton signals in the NMR spectrum, it was not possible to saturate the

20   His C_4 proton and obtain meaningful results. Indeed, the validity of the His C_2 saturation experiment is questionable and a control experiment was carried out to test the extent of partial saturation. Saturation of the Tyr(Me) meta protons resonance at $\delta=7.09$ ppm

25   (overlapped with the Phe ring protons resonance) resulted in enhancement of the Tyr(Me) ortho protons at $\delta=6.72$ ppm (30%) but not of the His C_2 and C_4 protons resonances. This experiment favors the absence of partial saturation effects contributing to the

30   enhancement of the Tyr(Me) meta and ortho protons resonances after saturation of the His C_2 proton, validating the experimental data showing proximity f th Tyr(M) and His rings of [Des[1]]Sarm sin in DMS .

-82-

Moreover, the interaction between the Tyr(Me) and His rings in [Des¹]Sarmesin is not a reverse relaxation phenomenon. Thus, while the His C_2 and C_4 protons can relax through the closely spaced Tyr(Me) meta and ortho

5   ring protons, the reverse effect is not observed upon saturation of the Tyr(Me) meta and ortho protons. The probable reason for this is that Tyr(Me) ortho and meta protons have relaxation pathways which are not available to the His C_2 and C_4 protons. The Tyr(Me)

10   ortho protons can relax through the Tyr(Me) meta and methyl protons, while the Tyr(Me) meta protons can relax through the Tyr(Me) ortho and C_a protons.

       In conclusion, the findings of this example suggest that Sarmesin and [Des¹]Sarmesin contain the

15   same bend at the His-Pro bond which has been observed for [Sar¹]Angiotensin II and that this produces similar clustering of the aromatic rings. Sarmesin and Angiotensin II appear to assume an approximately S-shaped conformation in DMSO. Previous work has

20   suggested that the N-terminus of [Sar¹]Angiotensin II interacts with the Tyr ring, whereas the present findings indicates that the N-terminus of Sarmesin is close to the His side-chain. From molecular modelling experiments, it can be shown that the Sar NCH_3 group

25   can occupy a position which is close to both the Tyr ring and the His C_a protons simultaneously.

-83-

Example 6 -- NMR Studies on Oxytocin and [Arg[8]] Vasopressin

NMR studies were carried out using a Bruker 400 Mz instrument essentially as described in previous examples. Peptides were dissolved at a concentration of 5mg/0.5ml of DMSO-d_6 and 2 drops of D_2O were added.

A characteristic resonance for the tyrosine hydroxyl proton at $\delta=9.2$ ppm was present in the proton NMR spectrum for vasopressin, but not in the NMR spectrum for oxytocin. The absence of this signal in oxytocin is diagnostic for tyrosinate formation and agrees with the fluorescence spectroscopy (Example 3); this signal was also absent for angiotensin II.

A ring pairing interaction for vasopressin is also evident when the NMR data for the Tyr and Phe rings of vasopressin are investigated. Theoretical calculations [Fowler et al., Biochem. Biophys. Res. Commun., 153(3), pp. 1296-1300 (1988)] have illustrated that electrostatic ring pairing interactions will occur preferentially in the perpendicular-plate orientation, and that a slipped parallel-plate configuration will only be adopted when other prevailing factors override perpendicular-plate interactions. In NMR experiments, perpendicular-plate interaction is accompanied by shielding of the protons of one ring and the absence of a shielding effect on the other ring, with both rings demonstrating non-equivalence of their ring protons. This is seen for vasopressin where the Tyr ring protons in the peptide are shielded (6.91 and 6.62 ppm) compared to the protons of free Tyr (7.07 and 6.70 ppm) or the Tyr ring protons of xytocin (7.12 and 6.68 ppm). The Phe ring protons of vasopressin are not

-84-

shielded and are non-equivalent (7.34 and 7.24 ppm)
compared to free Phe (7.30 ppm). This discloses the
fact that the hexagonal axis of the Tyr ring interacts
with the hexagonal face of the Phe ring in vasopressin.

5   The method can be used for any molecule where a ring
pairing interaction is possible.

        Using the charge distribution map depicted in
FIGURE 4B, together with the implicit considerations of
FIGURES 5 and 7, there are prepared the following new

10   antagonists to the Angiotensin II receptor, based on
modifications to the imidazole ring. It should be
noted that the nomenclature of the substituents of the
ringed compounds is different than that presented
above. Thus, the new antagonists to the angiotensin II

15   receptor are compounds of the formula:

$$R^{4A} - \underset{\underset{R^{4B}}{|}}{CH} - \alpha \bigcirc \begin{matrix} R^3 \quad R^2 \\ \epsilon + + \delta \\ \quad + - R^2 \text{ or } R^3 \\ \gamma \end{matrix}$$

$$\beta$$
$$\underset{\underset{R^{1A}}{|}}{CH-R^{1B}}$$

wherein $\alpha, \beta, \gamma, \delta$ and ϵ are C, N, O or S with the provisos that (a) the ring contains at least one C atom and one N atom, and (b) attachment of R groups is to C or N, and preferably further with the provisos that (c) at least one ring N atom remains unsubstituted, and (d) the pKa of the ring is $\leq$ 7 when all attendant groups have been taken into account;

R^{1A}, which mimics the structure in angiotensin of $- CH - CO - N - CH - CO -$

includes the following:

$-alk;$ $-O-alk;$ $-alk-O-alk;$ $-CH_2-CO-NH_2;$ $-CH_2-CO-NH-alk;$ $-CH_2-CO-N(alk)_2;$

$- CH_2 - CO - N \bigcirc alk$

$-CH_2-CO-AA-NH_2;$ or $CH_2-CO-AA-Phe,$ wherein AA is an amino acid preferably proline, azetidine-carboxylic acid, pipecolic acid, nipecotic acid, glycine, alanine, sarcosine, or N-methyl-alanine;

R^{1B}, which optionally provides a spacer arm terminating in a mimic of the C-terminal carboxylate group of angiotensin II, includes the following:

$-H;$ $-alk-A;$ $-\bigcirc^{A};$ or $-alk-\bigcirc^{A};$

preferably with the proviso that when R^{1B} is H, then: (a) if the ring is imidazole α and/or γ is other than N, (b) if the ring is other than imidazole either α is C or β is N, (c) R^{1A} comprises a group containing an amide, (d) R^2 comprises a group containing A, or (e) R^3 comprises a group containing B or $-Asp-Arg-NH_2;$

R^2, which provides steric and/or electronic properties and/or a spacer arm terminating in an acid group, includes the following: $-H,$ $-halide;$ $-alk;$

-86-

-O-alk; -NO$_2$; -CF$_3$; -CN; -alk-A; -A;

 ; or

R^3, which provides steric and/or electronic
properties and/or a mimetic of the tyrosine hydroxyl
group of angiotensin II in its "charge relay"
conformation, or a spacer arm terminating in a mimic of
the N-terminus of N-terminal dipeptide of angiotensin-
II, includes the following;

-H; -alk; -aryl; -alk-OH; -alk-halide; -CH$_2$-O-alk;
-CH$_2$-CN; -CH$_2$-CO$_2$H; -CH$_2$CO$_2$-alk; -NH-CO-alk;
-CO-NH-alk; -alk-B; -CH(OH)-alk-B; -alk-Asp-Arg-NH$_2$;
-CH(OH)-alk-Asp-Arg-NH$_2$;

 ; ; or ;

R^{6A}, which provides a spacer arm, the
relative rigidity of which is an aspect of the design,
terminating in an acid group which mimics the tyrosine
hydroxy groups of angiotensin II in its "receptor
bound" conformation includes the following:

 ,

 ,

 ,

 , and

-87-

where Z is a bond, -NHCO-, -O-, -OCH$_2$-, or -CH$_2$-;
X is -CO$_2$H, -alk-CO$_2$H, -PO$_3$H, -alk-PO$_3$H, -PO$_4$H$_2$, -alk-
PO$_4$H$_2$, -SH, -alk-SH, -SO$_3$H, -alk-SO$_3$H, -SO$_4$H$_2$, -alk-SO$_4$H$_2$,
F$_3$C-CO-NH-, F$_3$C-SO$_2$-NH-,

or yet another acid group
or a pharmaceutically acceptable salt thereof; and
Y is -H, -halide, -NO$_2$, -O-alk, -alk, -CF$_3$, or -CN; and
        R^{4B}, which optionally provides a spacer arm
terminating in a mimic of the N-terminus or N-terminal
dipeptide of angiotensin, includes the following:
-H, -alk-B, -alk-Asp-Arg-NH$_2$, alk-O-alk-B,
alk-O-alk-Asp-Arg-NH$_2$,

preferably with the proviso that when R^{4B} is H, then:
(a) if the ring is imidazole either α or γ is other
N, (b) if the ring is other than imidazole either α is
C or β is N, (c) R^{1A} comprises a group containing an
amide, (d) R^2 comprises a group containing A, or (e)
R^3 comprises a group containing B or is -Asp-Arg-NH$_2$;
        alk = an alkyl group having from 1 to 10
carbon atoms, a cycloalkyl group having 3-6 carbon
atoms, an alkenyl group having 2-10 carbon atoms, or an
alkynyl group having 2-10 carbon atoms;
        halide = -F, -Cl, -Br, or -I;
        A = an acid group or its pharmaceutical salt
and includes but is not limited to -CO$_2$H, -CO$_2$R$^+$,
-CO$_2$alk, -SO$_3$H, -SO$_4$H$_2$, -PO$_3$H, -PO$_4$H$_2$, F$_3$CCONH-, F$_3$CSO$_2$NH-,
    -alk-SH, or

-88-

wherein R' is a lipophilic ester prodrug form such as
$-CH_2CO_2C(CH_3)_3$ and the like;

B = a basic group or its pharmaceutical salt
including, but not limited to $-NH_2$, -NHalk, $-N(alk)_2$,
—N⟨◯⟩alk.

In a preferred aspect of the present
invention, when R^{1B} is H, then: (a) if the ring is
imidazole α and/or γ is other than N, (b) if the ring
is other than imidazole either α is C or β is N, (c)
R^{1A} comprises a group containing an amide, (d) R^2
comprises a group containing A, or (e) R^3 comprises a
group containing B or $-Asp-Arg-NH_2$.

In a further preferred aspect of the present
invention, when R^{4B} is H, then: (a) if the ring is
imidazole α and/or γ is other than N, (b) if the ring
is other than imidazole either α is C or β is N, (c)
R^{1A} comprises a group containing an amide, (d) R^2
comprises a group containing A, or (e) R^3 comprises a
group containing B or $-Asp-Arg-NH_2$;

In a particularly preferred product aspect of
the present invention, the above five-membered ring is
imidazole.
Preferably, there is no duplication of R^2 or
R^3 when not equal to H.
In the above formula, the group $-CH(R^{1A})(R^{1B})$
may be denoted R^1 and the group $-CH(R^{4A})(R^{4B})$ may be
denoted R^4. The relative positioning of the R groups
set f rth abov , and particularly the relati nship
between th R¹ group and th R⁴ group, giv s ris to

SUBSTITUTE SHEET

-89-

several different configurations in the case of five-membered rings such as imidazole, pyrole, pyrazole, triazoles, tetrazoles, thiazoles, etc.

For example, in the case of imidazole, the
following configurations apply:
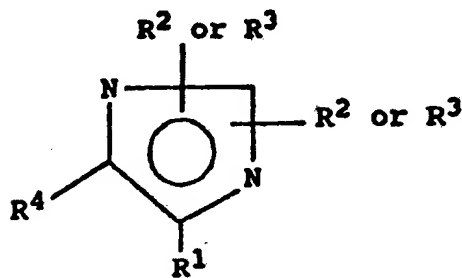
Configuration $N^{\gamma,\epsilon}$
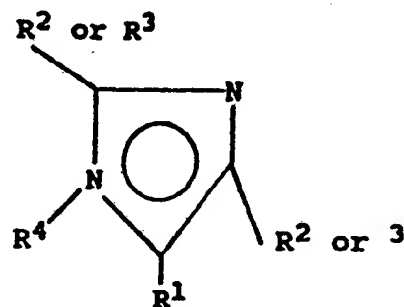
Configuration $N^{\alpha,\delta}$
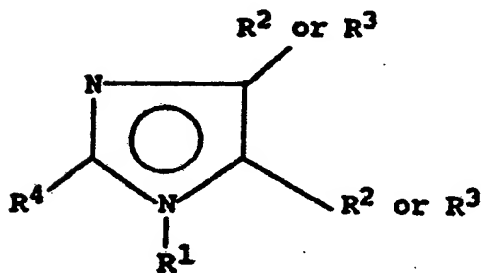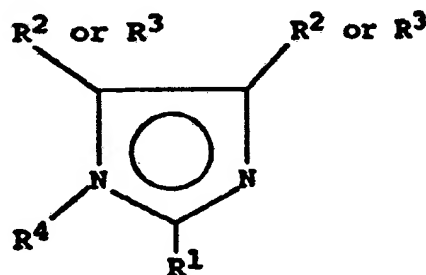
Configuration $N^{\beta,\epsilon}$
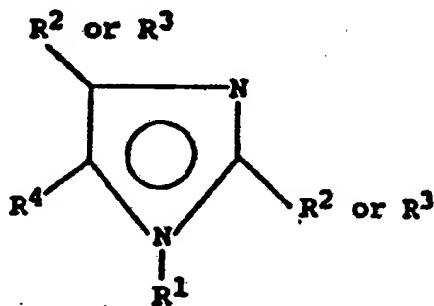
Configuration $N^{\alpha,\gamma}$

Configurati n $N^{\beta,\delta}$

-90-

Since R^4 is a substituted benzyl group or equivalent, configurations $N^{\alpha,\delta}$ and $N^{\alpha,\gamma}$ are generically speaking N-benzyl compounds, whereas configurations $N^{\gamma,\epsilon}$ and $N^{\delta,\epsilon}$, and $N^{\delta,\delta}$ are C-benzyl-imidazoles. Similar configurations, numbering ≤5 depending the number of ring N atoms present, apply to other five-membered heterocyclic rings.

The novel antagonists of the present invention are not limited to five-membered rings but indeed encompass six-membered rings including pyridine and diazines (such as, pyrimidine, pyridazine, and pyrazine) as well as triazines. For example, the following may serve as substitute for the imidazole ring in histidine:
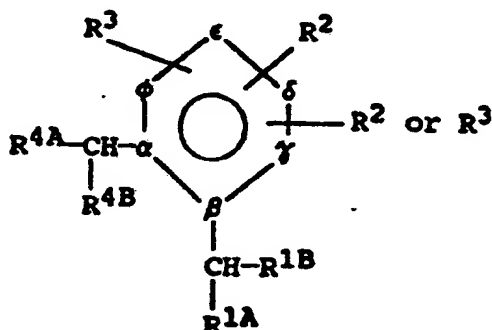
wherein α, β, γ, δ, ϵ, and ϕ are C, N, O or S with the provisos that (a) the ring contains at least one C atom and one N atom, (b) attachment of R groups is to C or N, (c) the number of substituted N atoms is one or more, and (d) the pKa of the ring is ≤ 7 when all attendant groups have been taken into account;

and wherein R^{1A}, R^{1B}, R^2, R^3, R^{4A}, Z, X, and Y, R^{4B}, alk, halide, A, and B are as defined previously.

Preferably, α is other than N.

Preferably, β is N.

-91-

Preferably, there is no duplication of R^2 or R^3 when not equal to hydrogen.

Yet another group of ringed moieties which may be substituted for the imidazole ring of histidine
5   are indoles, benzoazoles, and the like with the further proviso that steric considerations permit that such ring systems fall within the spatial constraints permitted by the conformational models of angiotensin set forth previously. Consideration of these
10   conformational models allows for three general configurations, as follows:
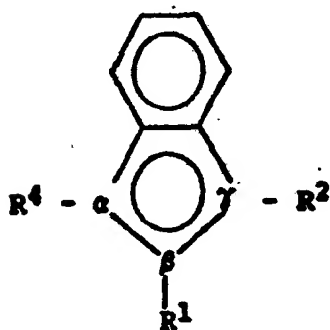
Configuration I
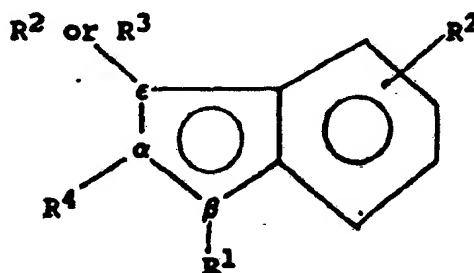
Configuration II
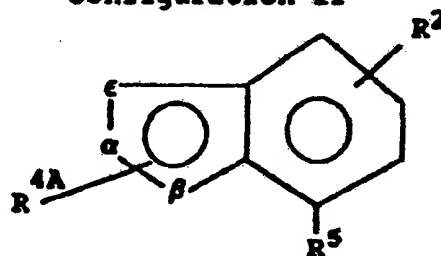
Configuration III

wherein α, β, and γ are C or N, with the proviso that only one N atom is substituted. R^1 is $-CH(R^{1A})(R^{1B})$ and R^4 is $-CH(R^{4A})(R^{4B})$. Substituents R^{1A}, R^{1B}, R^2, R^3, R^{4A}, R^{4B}

SUBSTITUTE SHEET

-92-

and R^5 are as defined above, except that for these
compounds when R^{1B} is H then (a) R^{1A} comprises a group
containing an amide, or (b) R^1 is on an N or (c) R^6 is
on a C. It is noted that configurations I and II apply
to indoles, but not to benzotriazole and benzopyrazole.
Configuration I applies to benzimidazole with the
proviso that no R^2 group is present at N^7, and
configuration II applies to benzimidazole with the
proviso that no R^2 or R^3 group is present at N^6.
Configuration III applies to indoles, benzimidazole,
benzopyrazole and benzotriazole; substitution of R^2 or
R^3 at ϵ is optional according to the above provisos
provided that only one nitrogen is substituted. $R^5=R^1$.

        Equivalent considerations apply to six-
membered heterocyclic ring systems such as
benzopyridine, benzodiazines, purines, quinolines,
phenanthrolines and the like.

        According to Figure 4 of the parent
application and considerations relating thereto, one
embodiment of the synthesis of new antagonists is based
on incorporating additional charges in appropriate
locations in BI and BABI compounds so as to increase
the binding affinity of these antagonists to the
angiotensin II receptor and accordingly increase their
potencies. Such considerations apply not only to N-
benzyl- and N-benzamidobenzyl-imidazoles but also to C-
benzyl- and C-benzamidobenzyl-imidazoles, as outlined
in the parent application and in further detail above
for 5 configurations of the imidazole ring:

SUBSTITUTE SHEET
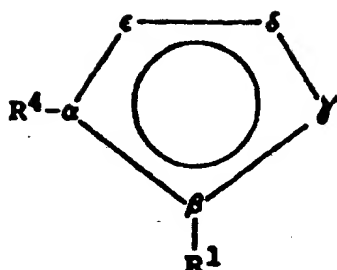
-93-

wherein R^4 = benzyl or benzamidobenzyl which is
optionally substituted and wherein α=N or C. According
to another consideration inherent to Figure 5 of the
parent application, the synthesis of new antagonists
also invokes the inclusion of an improved substitutent
to replace the n-alkyl group (R^1), namely, a more
accurate mimetic of the sequence $-CH_2-CH-CO-Pro-$

in angiotensin; such improved mimetics have been
outlined above and the following are particularly
relevant:

$-CH_2-CH_2-CO-NMe_2$; $-CH_2-CH_2-CO-N$; and
$-CH_2-CH_2-CO-Pro-NH_2$.
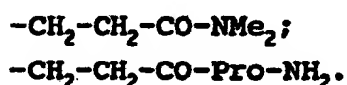
          In yet another consideration based on Figure
7 and in particular the discussion outlined on pages
43-46 of the parent application, the orientation of the
heterocyclic ring is an important aspect of the design
of angiotensin antagonists. As outlined previously,
five possible orientations or configurations, which are
determined by the placement of the N atoms in the ring
relative to the R substituents of the imidazole ring,
are applicable to the synthesis of angiotensin
antagonists. Furthermore, and as outlined previously,
ring systems isofunctional with imidazole can also be
present in these antagonists, including other azoles,
and including six-membered rings such as pyridin ,
diazines, and the like, as well as polynuclear ring

SUBSTITUTE SHEET

-94-

systems having at least one 5- or 6-membered
heterocyclic ring, as described above.

In a preferred embodiment of the present
application, the heterocyclic ring is imidazole in any

5   of its five possible configurations outlined
previously. A particularly preferred embodiment is
configuration $N^{\tau \cdot \iota}$ which gives rise to C-benzyl-
compounds and which exactly mimics the imidazole group
of the histidine residue of angiotensin-II (See figure

10   5). Furthermore, it is also a preferred embodiment
that the R^1 substituent is not a straight chain
hydrocarbon but contains an amide function mimicking
the His-Pro group in angiotensin II. In yet another
preferred embodiment, a spacer arm terminationg in a

15   charged group, which mimics the N- or C- terminus of
angiotensin II, is incorporated at R^2 or R^3 or R^1B or
R^{48}, as outlined previously. The criteria for these
preferred embodiments are based on molecular modelling
of angiotensin as outlined in Figures 4,5, and 7 and

20   relevant discussion thereto, including pages 43-46 of
the parent application.

The compounds depicted above can be readily
prepared by the skilled artisan using art recognized
techniques. Such compounds and their pharmaceutically

25   acceptable salts are useful as Angiotensin II
antagonists. Accordingly, such compounds can be used
to control hypertension and/or congestive heart failure
in a mammal in need of such treatment. Additionally,
the compounds of this invention are contemplated as

30   being useful in other cardiovascular and related
diseases such as stroke, myocardial infarction and the
like. When used to control hypertension and/or

SUBSTITUTE SHEET

-95-

congestive heart failur , the compound is normally
administered to such a mammal either orally or
parenterally. When so administered, the compound is
generally formulated in a pharmaceutically acceptable
5   diluent and at a dosage sufficient to control
hypertension and/or congestive heart failure in the
mammal so treated. The specific dose levels for such
uses can be readily determined by the skilled artisan.
Accordingly, the present invention contemplates a
10   method for controlling hypertension in a mammal in need
of such treatment which comprises either administering
orally or parenterally a pharmaceutical composition of
a compound depicted above in an amount sufficient to
control hypertension. Additionally, the present
15   invention also contemplates a method for treating
congestive heart failure in a mammal in need of such
treatment which comprises either administering orally
or parenterally a pharmaceutical composition of a
compound depicted above in an amount sufficient to
20   control said heart failure. The methods of controlling
hypertension are implemented using pharmaceutical
compositions comprising a pharmaceutically acceptable
carrier and an amount of a compound depicted above
effective to control hypertension in a mammal in need
25   of such treatment. The methods of controlling
congestive heart are implemented using pharmaceutical
compositions comprising a pharmaceutically acceptable
carrier and an amount of a compound depicted above
effective to control said heart failure.

30   Methods of preparing the above-described
compounds are now described. The synthesis of
heterocyclic compounds follows methods well known to
on skill d in the art, such as methods d scribed in

-96-

Comprehensiv H terocyclic Chemistry, P rgamon Press,
New York, wherein Vols 4 and 5 (1984) are particularly
relevant to the present invention. Synthetic methods
of the present invention have also been reviewed in
detail in European Patents 0263310 and 0323844. In
view of the knowledge in the art concerning the
synthetic routes employed, general synthetic schemes
for the preparation of compounds according to the
invention are presented below.. Such schemes generally
utilize combinations of chemical transformations
together with strategies and protecting groups familiar
to one skilled in the art.

          Unless otherwise stated, all reactions are
conducted at temperatures ranging from 20°C to the
reflux temperature of the solvent for between two hours
and two days in a suitably inert solvent such as
dimethylformamide, dimethylsulfoxide, chloroform,
methylene chloride, benzene, toluene, dioxane,
tetrahydrofuran, or ether.

Synthesis of Substituted Imidazoles
          The following references are pertinent to the
synthesis of substituted imidazoles. Advances in
Heterocyclic Chemistry, Vols. 4,12,27,35 (Cambridge
University Press) and Heterocyclic Nitrogen Compounds:
The Azolesz, K. Schofield et al (1976) Cambridge Univ.
Press. A general scheme for the synthesis of
substituted imidazoles involves condensation of an
amidine or related compound with an α-halo/hydroxy-
ketone:

-97-
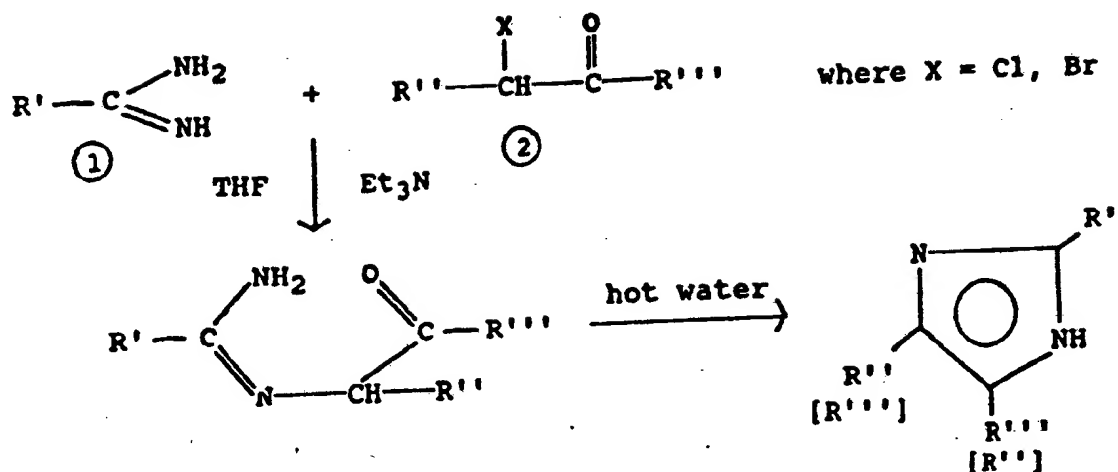
The isomers are separated by conventional methods such as crystallization or chromatography.

The amidine 1 is prepared from the nitrile directly or via the iminoether:

5
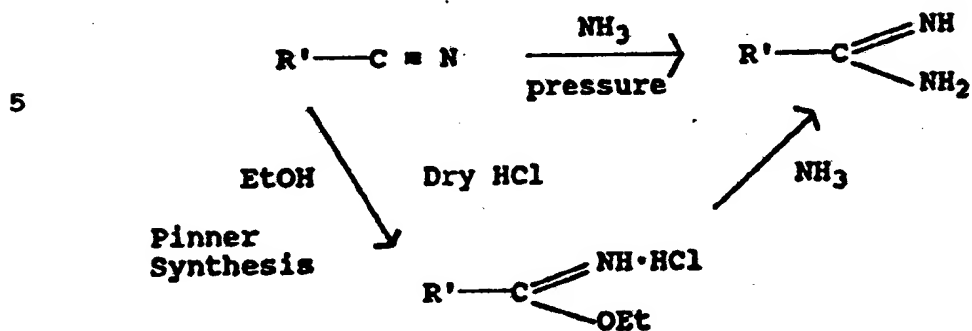
-98-

The halo/hydroxy-ketone 2 is prepared by numerous methods known in the art, for example:

5 (A) $R'''-CO_2H$ $\xrightarrow[\text{diazomethane/ether}]{\text{dicyclohexylcarbodiimide}}$ $R'''-\overset{\overset{\displaystyle O}{\|}}{C}-CHN_2$

10 $\xrightarrow[\text{N-chlorosuccinimide dioxane}]{\text{2 equiv.}}$ $R'''-\overset{\overset{\displaystyle O}{\|}}{C}-CHCl_2$

$\downarrow (R'')_2CuLi$

15 $R''-\overset{\overset{\displaystyle Cl}{|}}{C}H-\overset{\overset{\displaystyle O}{\|}}{C}-R'''$

(B) $R''-CH_2CHO$ $\xrightarrow{\text{1,3 propane dithiol}}$ $R''-CH_2-\underset{S}{\overset{S}{<}}$

1) BuLi/THF
2) R'''I

20 $R''-CH_2-\overset{\overset{\displaystyle O}{\|}}{C}-R'''$ $\underset{H^+}{\longleftarrow}$ $\underset{R'''}{\overset{R''-CH_2}{>}}\times\underset{S}{\overset{S}{<}}$

$H^+ \downarrow$ 1 equiv. N-chlorosuccinimide

25 $R''-\overset{\overset{\displaystyle Cl}{|}}{C}H-\overset{\overset{\displaystyle O}{\|}}{C}-R'''$

1) BuLi/THF
2) R'''CHO
3) Hg^{2+}

$R'''-\overset{\overset{\displaystyle HO}{|}}{C}H-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-R''$
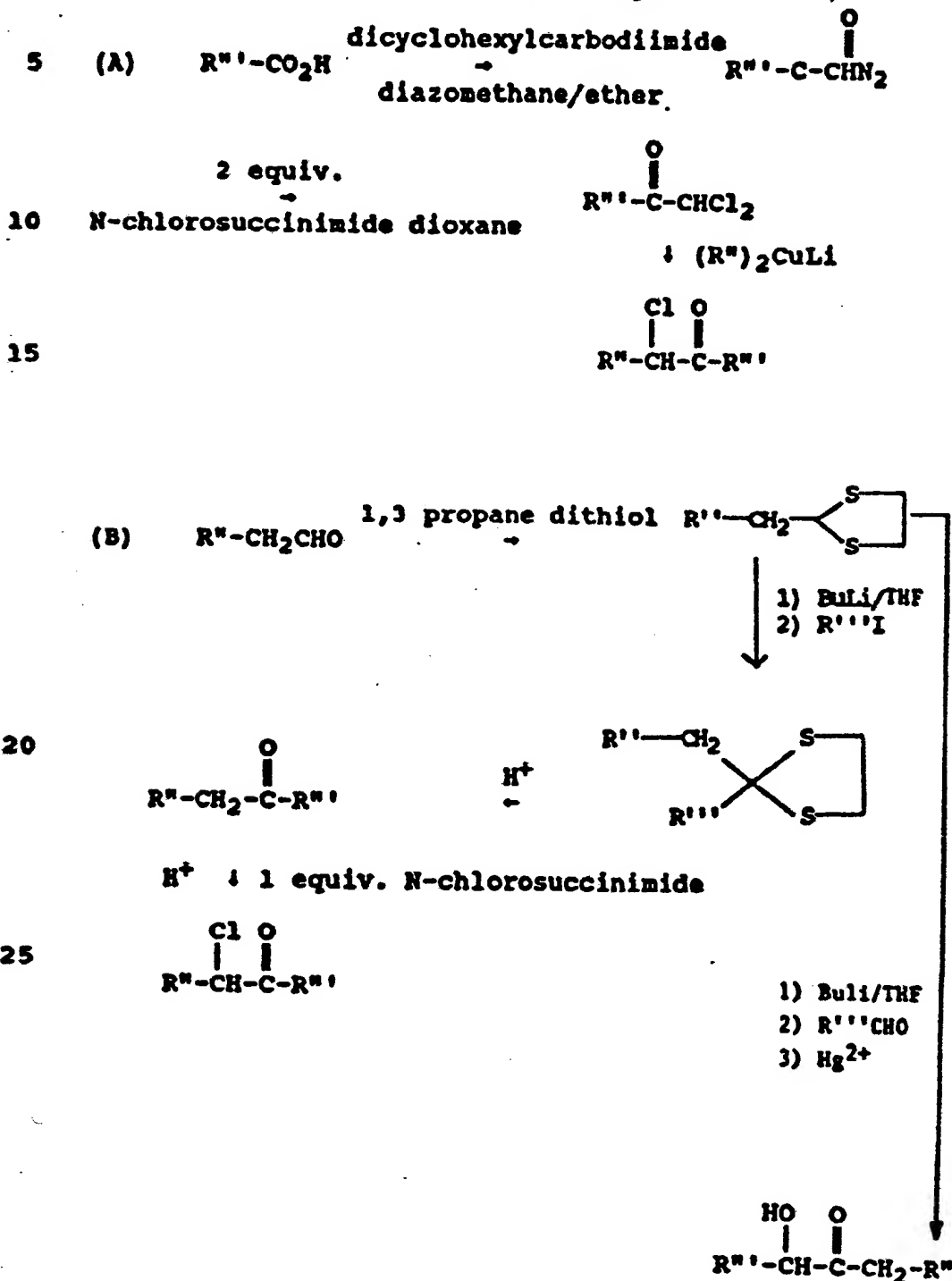
SUBSTITUTE SHEET

-99-

Alternatively, an iminoether can be condensed
with an α-hydroxy/halo-ketone in the presence of
ammonia:
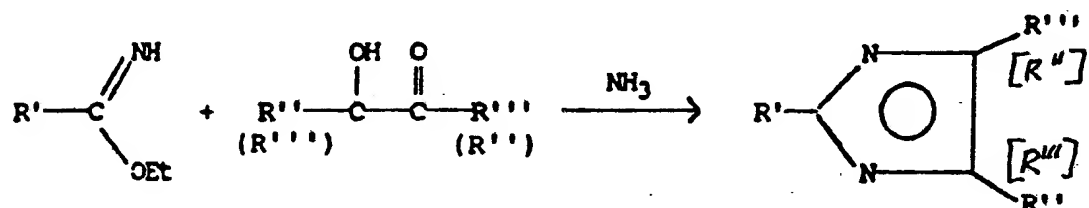
5   The isomers are separated by crystallization
or chromatography.

         In another method applicable to substituted
N-benzyl compounds in particular, iminoether is reacted
with substituted benzylamine to form the amidine which
10   is subsequently condensed with α-halo- or α-hydroxy-
ketone/aldehyde:
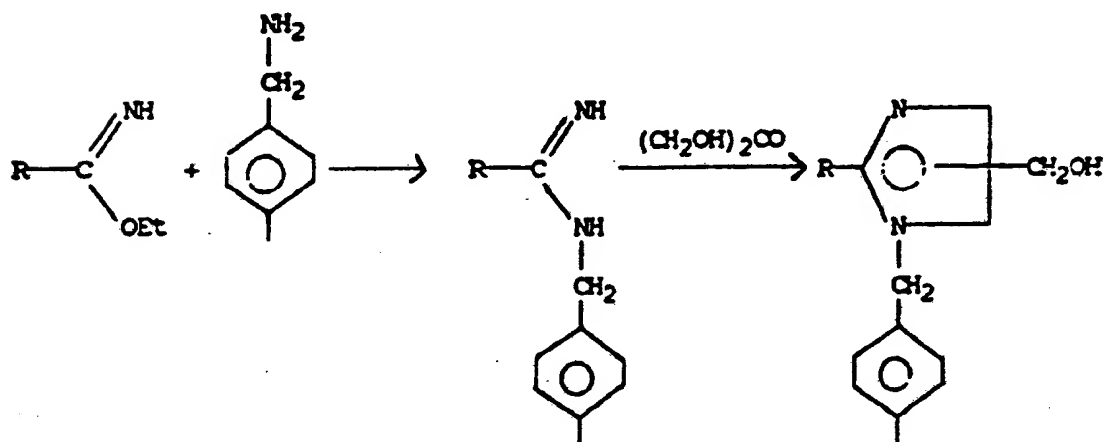
-100-

In yet another method applicable to the synthesis of substituted N-benzyl compounds, acylaminoketone is reacted with derivatized benzylamine to form an imine which is then converted to N-benzylimidazole:

5
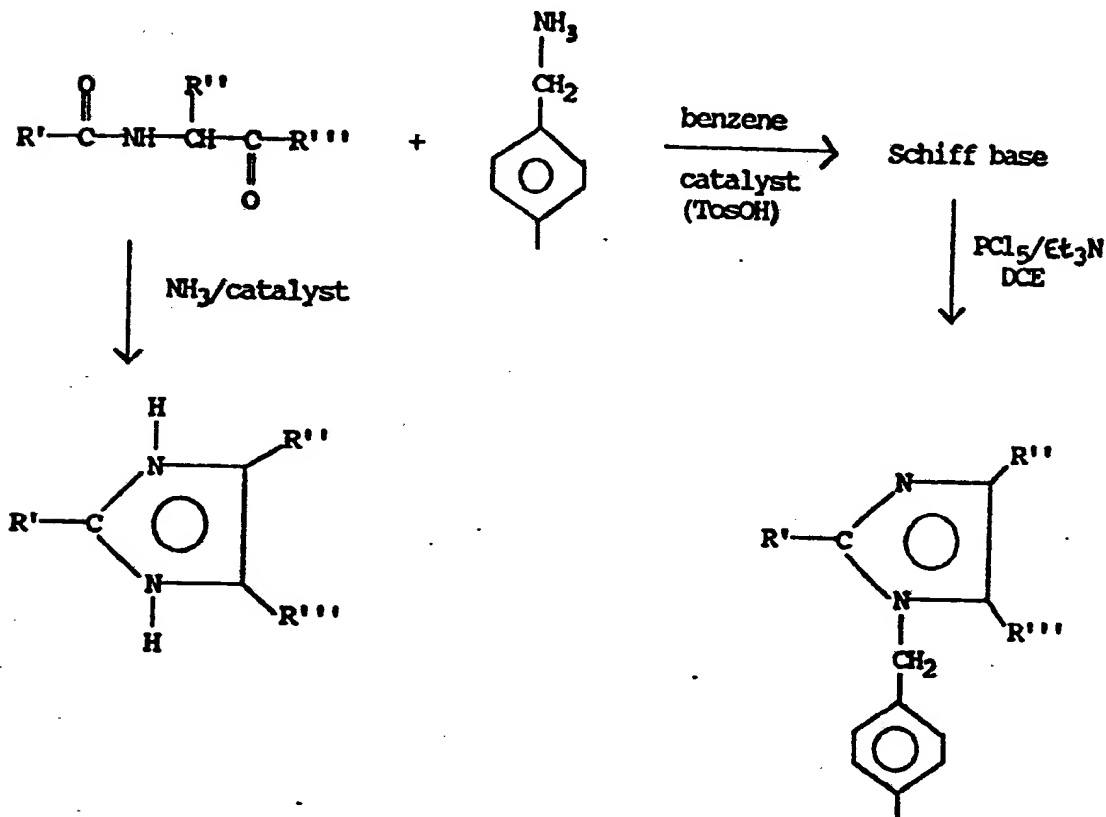
Likewise, imidazole compounds can be obtained using ammonia instead of amine as set forth in Davidson et al, *J. Org. Chem*, *2*, 319, 1937 and Heinze et al, *Chem. Ber.* *101*, 3504, 1968.

10   Acylaminoketone is readily obtainable from amino acids using the Dakin-West reaction and modifications thereof as well as from the corresponding α-haloketone by art recognized methods.

-101-

Preparation of Acylaminoketones

Aminoacylketones of the general formula

$$R'-CO-NH-\underset{\underset{R''}{|}}{CH}-CO-R'''$$

can be prepared frim N-acyl amino acid by reaction with alkyllithium or alkylcopperlithium:
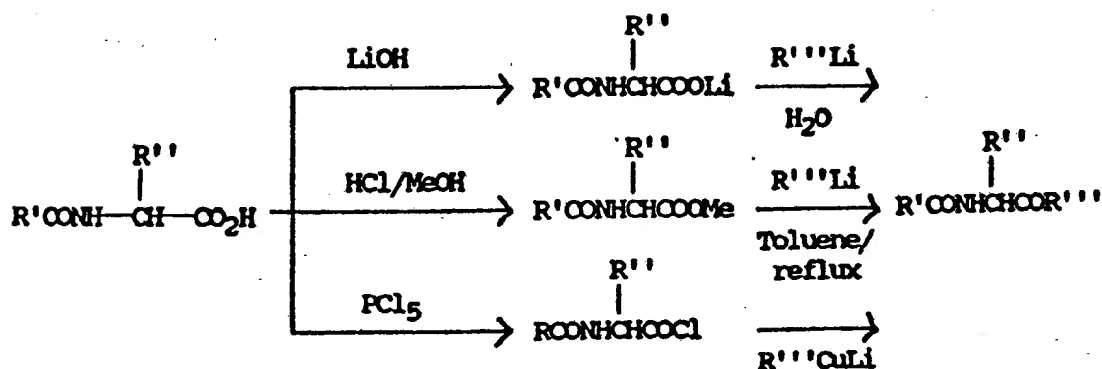
For example, N-acyl-DL-4-nitrophenylalanine can be converted to its butylketone derivative using, e.g., butyllithium.

Another general method for preparing acylaminoketones is by the Dakin-West reaction in which the acyl amino acid is converted to the required acylaminoketone by reaction with anhydride in the presence of base (See Hofle et al, Angew. Chem. Int. Ed. Vol. 17, p. 569, 1978):

$$R'-CO-NH-\underset{\underset{R''}{|}}{CH}-CO-OH \quad \xrightarrow[\text{Pyridine}]{(R'''CO)_2O} \quad R'-CO-NH-\underset{\underset{R''}{|}}{CH}-CO-R'''$$
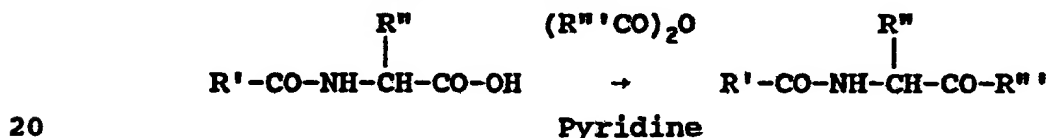
This reaction proc eds through th oxazolinon , and provid s an alt rnative stepwise

-102-

approach for preparing acylaminoketones when the amino
acid is not readily available. For example,
acylglycine can be converted to other acylamino acids
by alkylating the oxazalinone intermediate as follows:

[R' = trifluoromethyl, phenyl, alkyl, etc.]

oxazalone
(oxazalinone)

1 equiv. R''X
Et$_3$N   (X = Br, I)
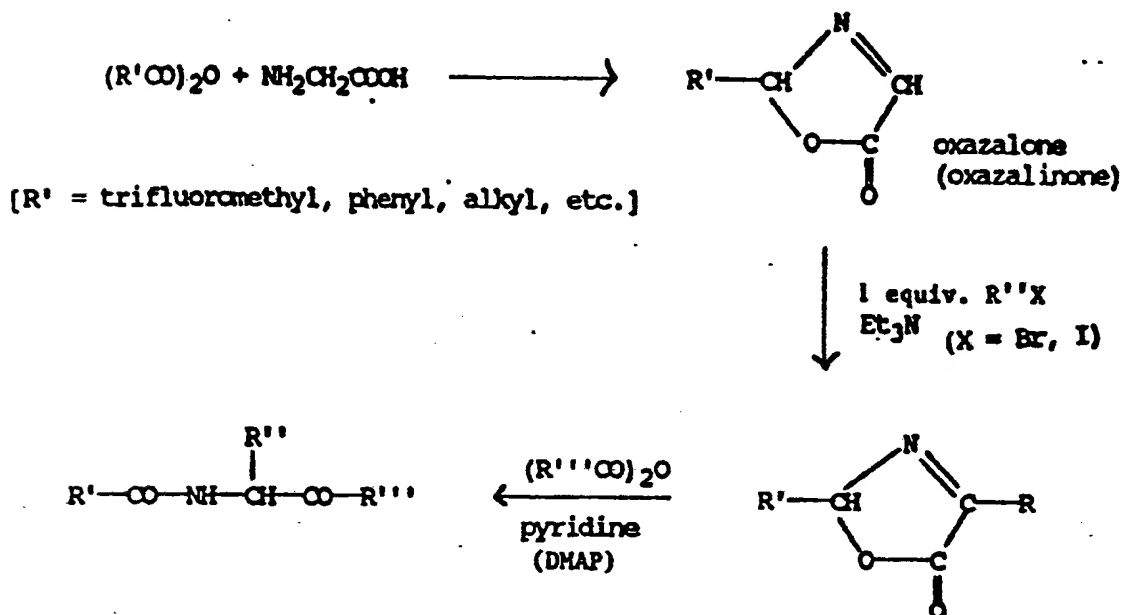
5   For N-benzyl compounds, alkylation of
imidazole nitrogen can be carried out as follows:
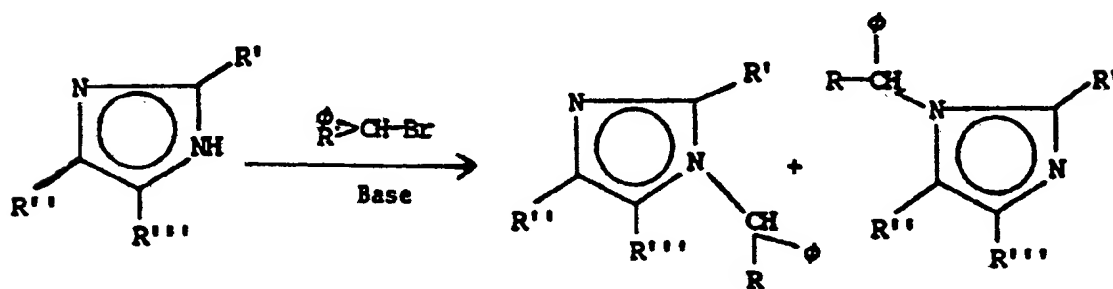
The two products can be separated by
classical methods such as crystallization and
chromatography.

-103-

For C-benzyl compounds, the imidazole
nitrogen can be protected with a suitable protecting
group such as tosyl, benzyloxymethyl, trityl, or
benzyl, which can be subsequently removed by a

5   strategically acceptable method such as acidolysis or
hydrogenation. If the protecting group is to be
removed at the end of the synthesis, the two products
formed do not need to be separated:

(2 products)
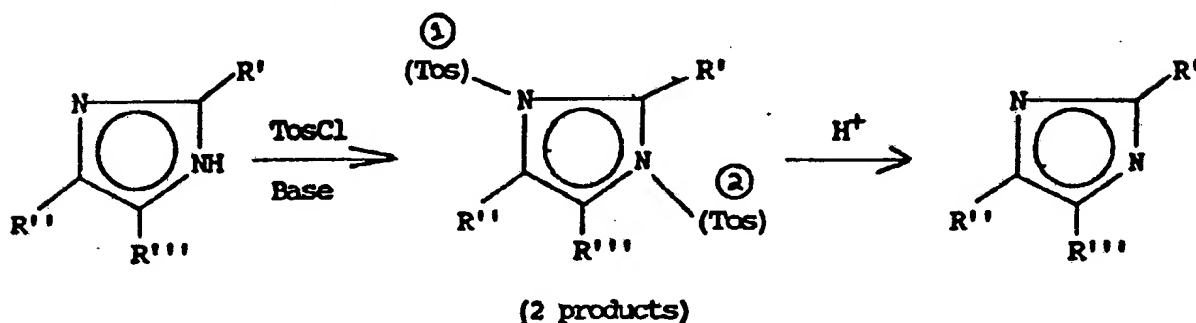
As previously indicated, it is considered to.

10   be within the skill of persons in the art to prepare
compounds as described. One skilled in the art knows,
for example, that it is often not possible to introduce
a side group during synthesis in the form which is
required in the final product. Thus, for example, an

15   amino group often cannot be introduced in the middle of
the synthesis scheme because its reactivity towards
electrophiles is high and it could therefore become
irreversibly modified during the course of the
synthesis to the final product. The amino group is

20   therefore introduced as a nitro group or as an
acylamino group or in yet another form in order to
circumvent this problem. At or near the end of the
synthesis scheme, at which point conversion to an amino
group will not compromise the integrity of the final

25   product, th amino gr up is produced using classical
chemical procedur s. Similarly, when the end product

-104-

is to contain a carboxylate function, it is introduced in the form of a nitrile, alcohol, ether, ester, alkene or some other art-recognized precursor.

Conversions of this general type are done by the classical reactions shown below, and produce the side-groups indicated. For further details, see J. March, Advanced Organic Chemistry, 1985 J. Wiley & Sons, New York and references therein.

1. Carboxylate:
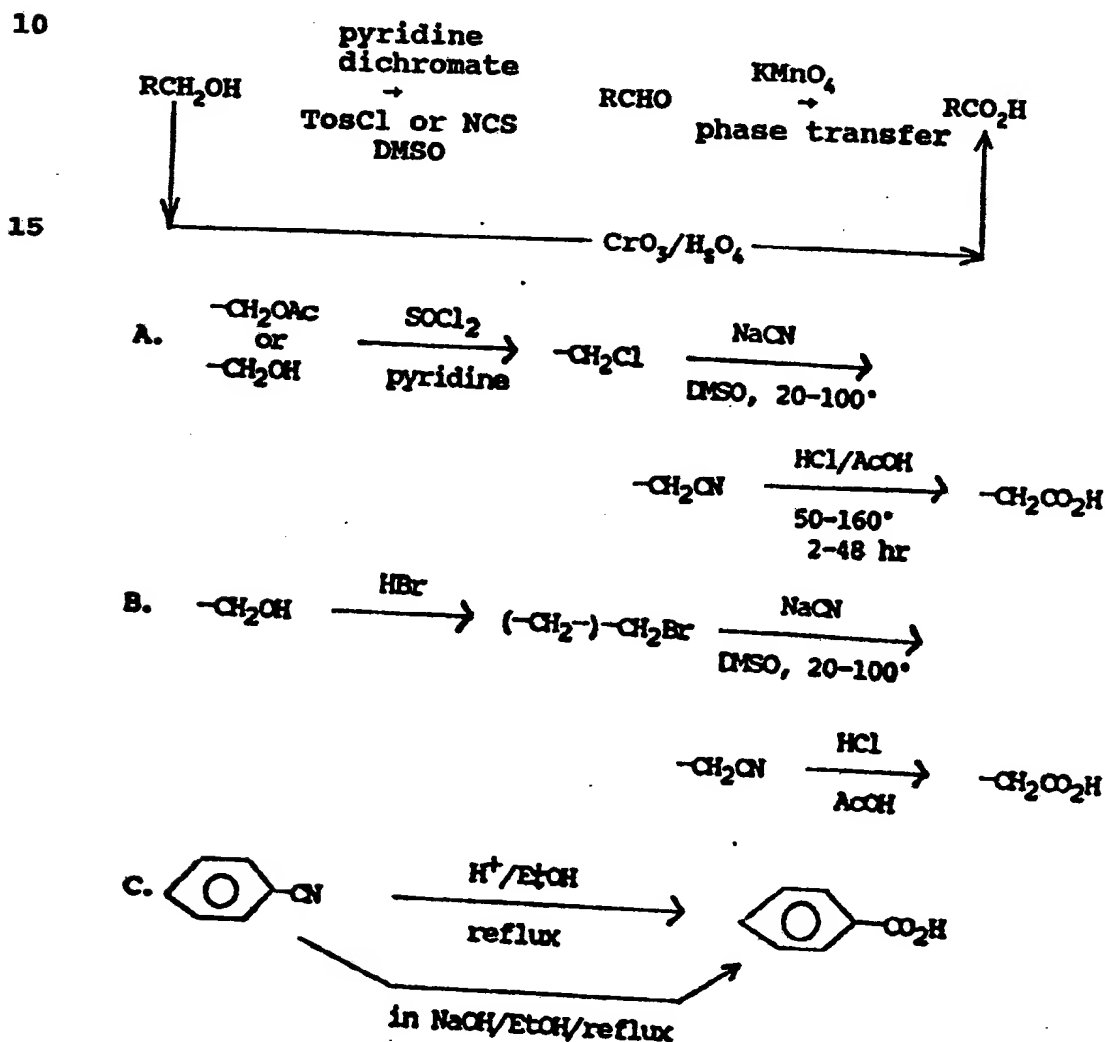
SUBSTITUTE SHEET

-105-

2. Amino

NO_2—⟨benzene⟩— →[H_2/Pd / or Fe/AcOH / or Zn/AcOH]

NH_2—⟨benzene⟩— →[0-5° NaNO₂ / HCl] N_2-Ar →[Sandmeyer 50° CuCN / KCN] CN—Ar →[HCl / AcOH] CO_2H-AR

N_2-Ar →[CuX₂ 15-60° Sandmeyer] X — Ar   where X = halide
                                              Ar = aryl

B.   —NH(CO-O-^{t}Butyl) →[TFA]   —NH_2

Boc(protecting group)

3. Tetrazole

R-CN →[1. SrR₃N₃ / 2. H⁺ or OH⁻] R-C⟨tetrazole ring N-N / N-N / H⟩

R-CN →[NaN₃, 30-100° / NH₄Cl, DMF] R-C⟨tetrazole⟩

4. Ether

—CH_2OH →[HCl/MeOH / reflux] —CH_2OCH_3

—CH_2OH →[1. NaOEt or NaH / 2. RX] —CH_2OR —CH_2Cl →[NaOR / DMF] —CH_2OR

SUBSTITUTE SHEET

-106-

5. Ester

$$-CH_2CO_2H \xrightarrow[\text{reflux}]{\text{HCl/MeOH}} -CH_2CO_2CH_3$$

$$-CH_2OH \xrightarrow[\text{Et}_3\text{N or pyridine}]{\text{Ac}_2\text{O}} -CH_2OAc$$

6. Amide

Dicyclohexylcarbodiimide

1-Hydroxybenzotriazole

SOCl$_2$
pyridine

anhydride

R$_2$NH

RNH$_2$
OH$^-$

-CONHR

or -CONH

7. Halogenation

N-halosuccinimide
1-2 equiv., dioxane

Dehalogenation
H$_2$/Pd

-107-

8. Sulfonation/Phosphonation
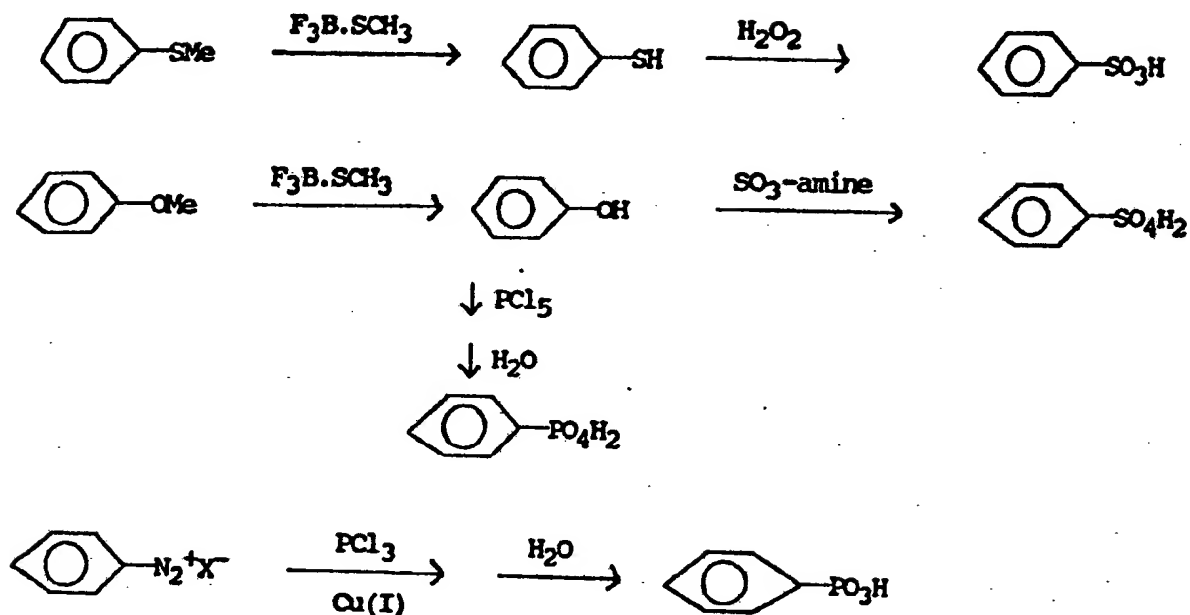
9. Trifluoromethylsulphonamido and Trifluoromethylacet-amido
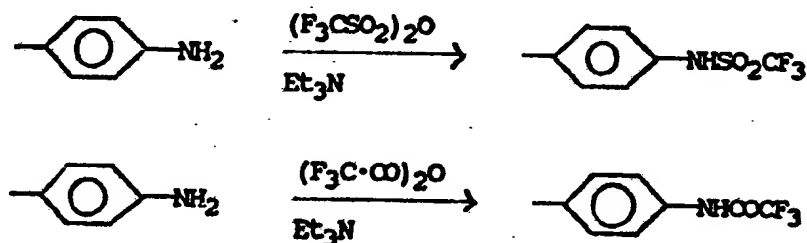
5   ## 10. Protecting Groups for -NH, -NH$_2$, -OH, -CO$_2$H, -CONH$_2$, or
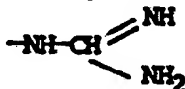
Acid-labile and base-labile protecting groups
and protecting groups removable by hydrogenation are
well known in the art. Methods for introducing such
10   protecting groups and for their removal are familiar to
one skilled in the art as set forth in J. Stewart and
J. Young, <u>Solid Phase Peptide Synthesis</u>, 1984, Pierc
Chem. Co.

-108-

11. Nitro and Trifluormethyl
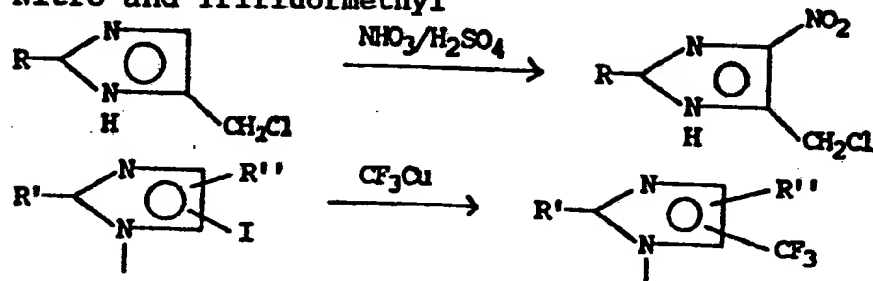
12. $-(CH_2)_n-Asp-Arg-NH_2$
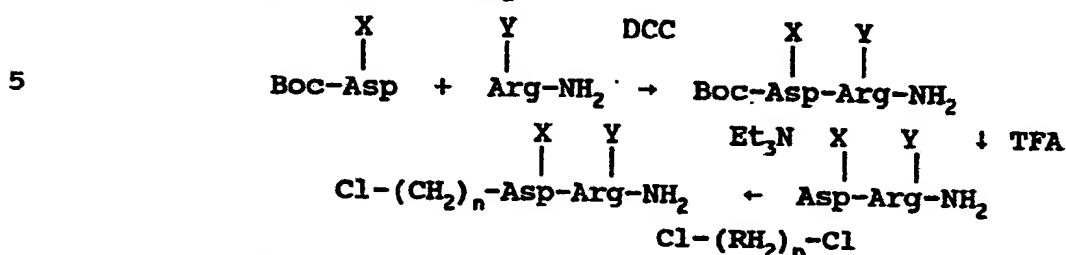
where X=benzyl, t-butyl   Y=tosyl, nitro

In the above reaction, peptide should be added to large excess of cross-linker to avoid dimerization.

Non-imidazole Compounds

As previously indicated, it is possible to employ non-imidazole compounds as new antagonists. Such compounds are prepared by procedures which are analogous to those described above which provide substituents on the ring which can be converted to other groups. Details of methods and strategies for these syntheses have been extensively reviewed in European Patent 0,323,841 for compounds similar to the compounds of the present invention. Consequently, the following is a summary of the more important strategies available to obtain the required compounds, and is not intended to cover the entire field. As with substituted imidazoles, the elaboration of heterocyclic rings derivatized at the ring produces often a mixture of products which can be separated by conventional

-109-

chromatography methods, and the isomers individually identified by Nuclear Overhauser Effect spectroscopy, and in some cases by bioassay or binding assay, i.e., the ability to displace angiotensin II from its

5   receptor site.

Substituted Pyrole

1. Paal-Knorr reaction: condensation of 1,4 dicarbonyl compounds with ammonia or primary amine, as follows:

$$R'CO \cdot CH_2 \cdot CH_2 \cdot CO \cdot R'' \quad \xrightarrow{R'''NH_2} \quad \xrightarrow{H^+}$$

$$\xrightarrow{NH_3} \quad \xrightarrow{R'''X}$$

(product: pyrrole ring, R' — ring — R'', N-R''')   [X=halide]
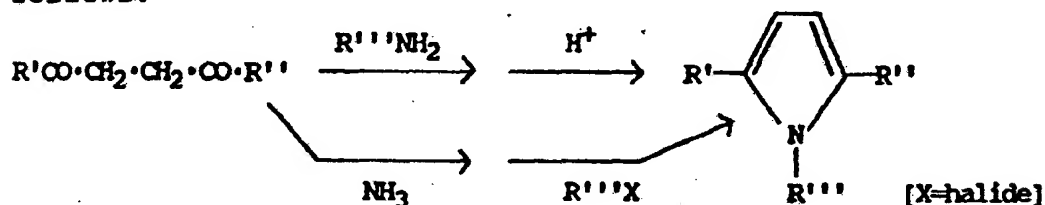
10   2. Hantzsch synthesis: condensation of α-haloketones (or α-hydroxyaldehyde or nitroalkenes) with β-ketoesters in the presence of ammonia:

$$R' \cdot CO \cdot CH_2 \cdot X \;+\; R'' \cdot CO \cdot CH_2 \cdot CO \cdot OEt \quad \xrightarrow{NH_3}$$

(product: pyrrole ring bearing CO·OEt, R' — ring — R'', N-H)

(lower product: pyrrole ring bearing CO·OEt, R' — ring — R'', N-R''')

$$\xleftarrow{R'''X}$$

[X = halide]
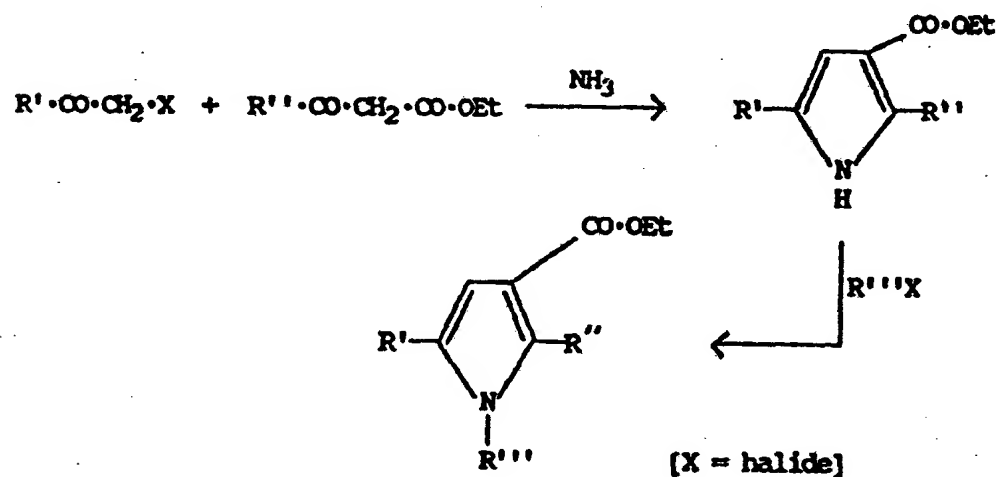
Substituted Pyrazole:

Condensation of 1,3 dicarbonyl compounds with

15   hydrazine or its derivatives:

-110-

$$R'CO \cdot CH_2 \cdot CO \cdot R'' \xrightarrow{\ R'''N_2H_4\ }$$

[X=halide]

$$\xrightarrow{N_2H_4} \xrightarrow{R'''X}$$
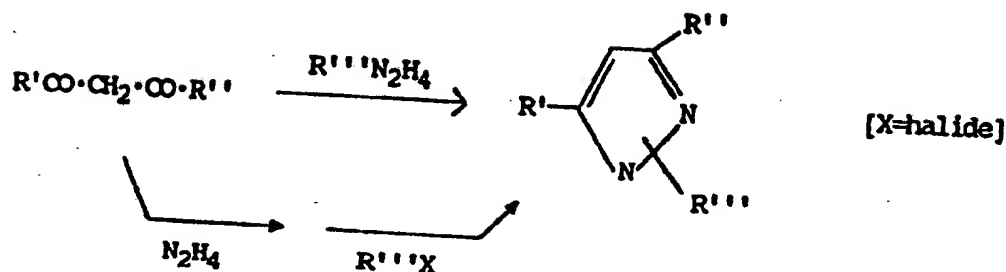
Substituted 1,2,3-triazole

Thermal cycloaddition of azides to alkynes:

$$R'-C\equiv CH \xrightarrow{\ R''N_3\ } \xrightarrow{\ H^+\ }$$

[1,4 substitution is favored but 1,5 isomer also formed]

$$\downarrow NH_3$$

$$\xrightarrow{R''X}$$ mixture of 1-, 2-, and 3-substitution   [X=halide]
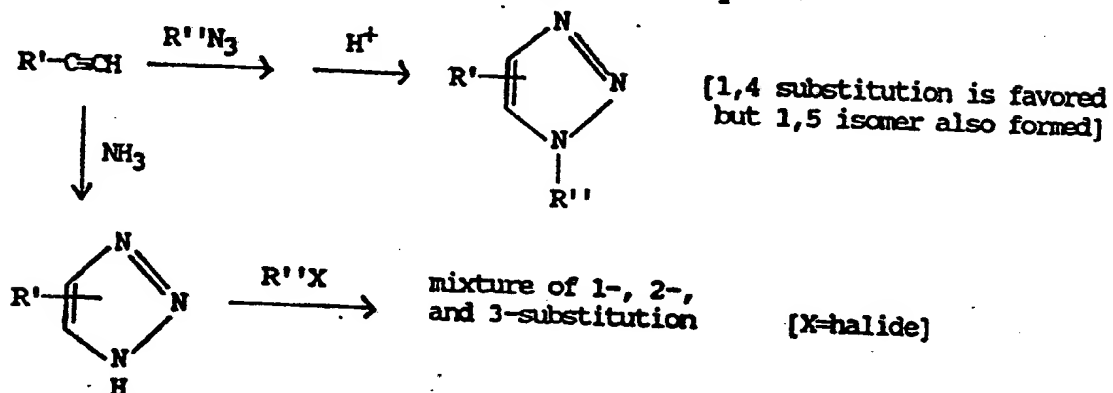
5 ### Substituted 1,2,4-triazole

Reaction of orthoester and acylhydrazine to give 1,2,4-oxadiazole followed by reaction with ammonia or primary amine:

$$R'C(OR)_3 \ + \ R''CO \cdot NH \cdot NH_2 \longrightarrow$$

$$\swarrow NH_3 \qquad \searrow R'''NH_2$$

mixture of 1- and 2-substitution $\xleftarrow{\ R'''X\ }$

[X=halide]
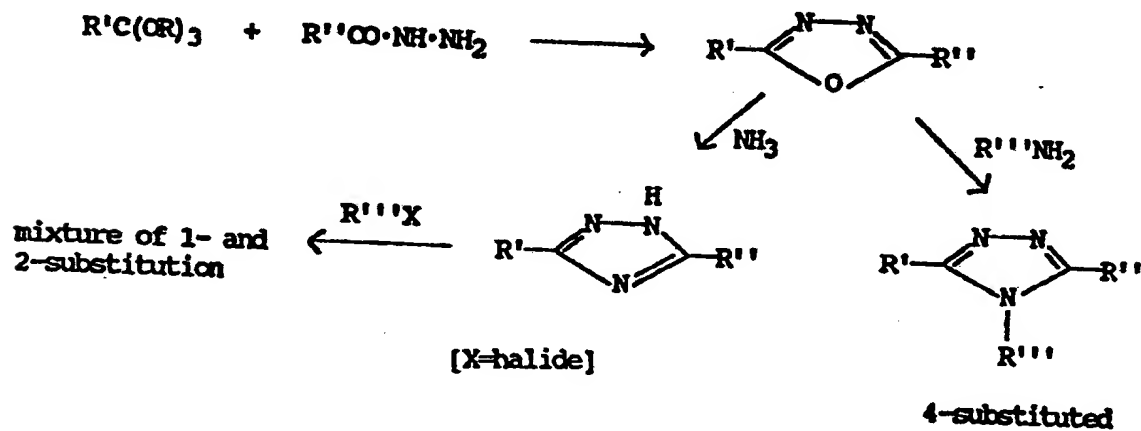
4-substituted

-111-

Substituted Tetrazole

General methods elaborated previously may be applied as set forth concerning protection of tetrazole:
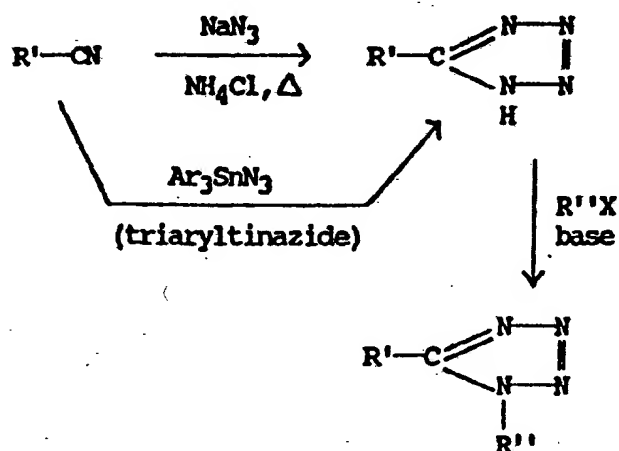
Protection of tetrazole may be accomplished using a trityl group if required or it may be otherwise substituted accordingly. [X=halide].

5   Substituted Pyridines, Diazines and Triazines are produced by analogous methods.

-112-

<u>Polynuclear Heterocyclic Compounds</u>
<u>Substituted Indoles, Benzimidazole, Benzopyrazole and</u>
<u>Benzotriazole</u>

5   Fischer indole synthesis: arylhydrazones of aldehydes or ketones as treated with a catalyst such as $ZnCl_2$
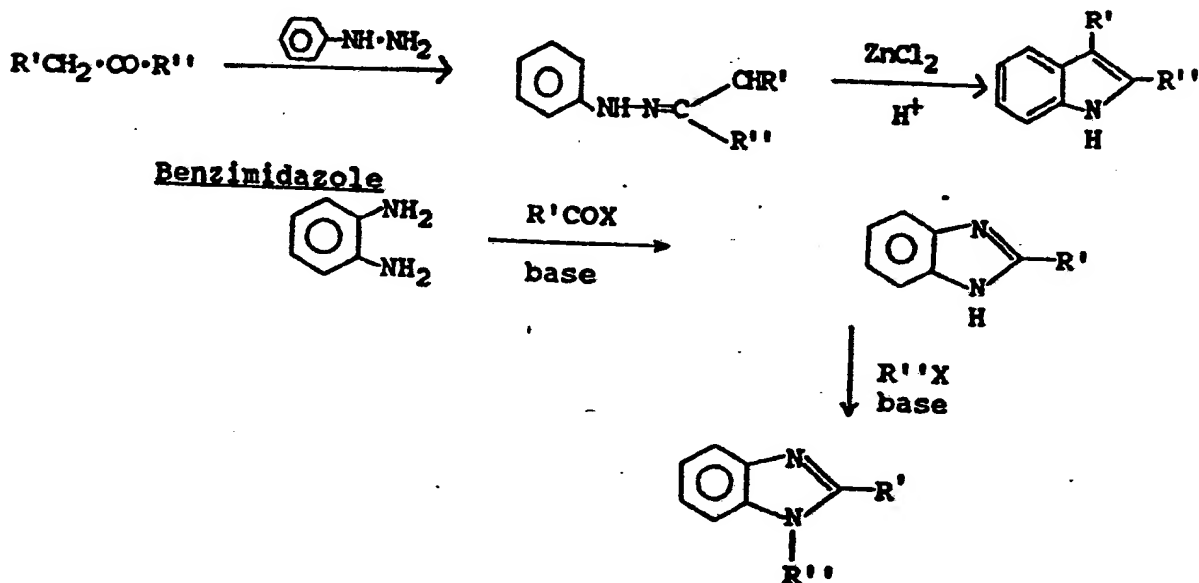
-113-

Benzopyrazole

5

10

15

Benzotriazole

20

25

30
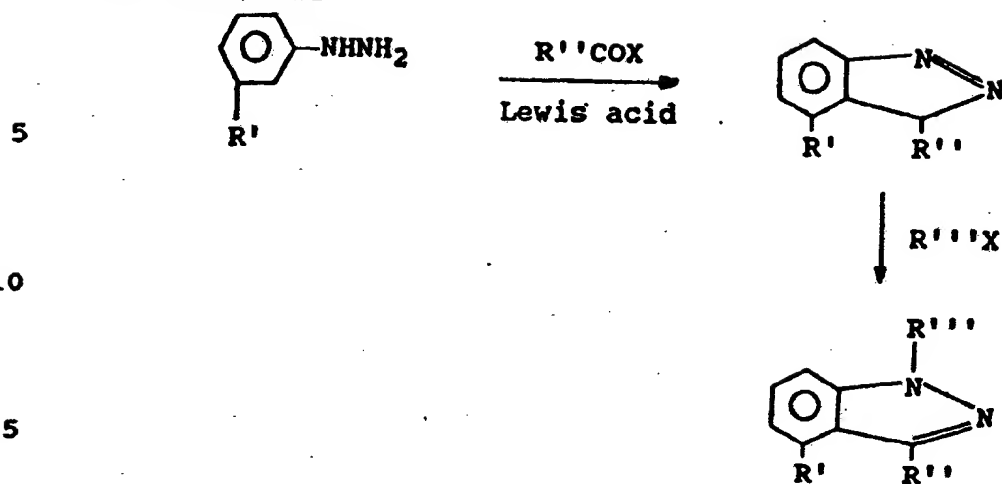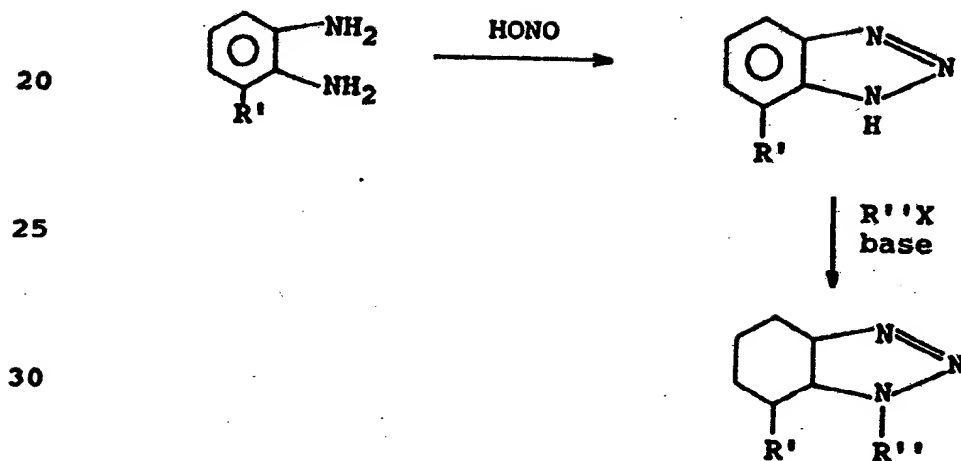
-114-

Substituted Benopyridines, Benzodiazines and Benzotriazines are produced by analogous methods.

Compound Synthesis

5   The choice of starting materials and strategy of synthesis of a given compound is dictated by a number of factors including feasibility and cost. Th choice of methods and protecting groups and precurs r groups for a given synthesis is largely determined by
10   other compromising structural and chemistry factors, which can be satisfactorily ascertained by one skilled in the art.

SUBSTITUTE SHEET

-115-

WHAT IS CLAIMED IS:

1.   A method for creating a three-dimensional spatial model for a biologically active ligand having one or more active sites based on a charge-transfer interaction and further having a known structural formula wherein the three-dimensional spatial assignments for each of the atoms of the ligand in the model are assigned from the steps comprising:

        a)   determining the presence of charge-transfer interaction(s) in said ligand from fluorescence analysis of said ligand in a fluorescence compatible environment;

        b)   determining the chemical groups involved in said charge-transfer interaction(s); and

        c)   resolving remaining aspects of the ligand's three-dimensional conformation by obtaining conformational information relative to the active site(s) from nuclear magnetic resonance spectroscopy employing the nuclear Overhauser effect providing that when the nuclear Overhauser effect technique employed in this step is NOESY, then the molecular weight of said ligand is either less than about 500 or greater than about 2000.

2.   A method according to Claim 1 which further comprises refining the three-dimensional model so generated by use of theoretical considerations.

3.   A method according to Claim 1 which further comprises creating new three-dimensional models for said ligand by use of theoretical consideration.

-116-

4. A method according to Claim 1 wherein said biologically active ligand is selected from the group consisting of Angiotensin II, oxytocin, and vasopressin.

5   5. A method according to Claim 1 wherein said charge-transfer interaction is a tyrosinate charge-transfer interactions.

6. A method according to Claim 5 wherein said ligand has one active site based on a tyrosinate 10   charge-transfer interaction.

7. A method according to Claim 6 wherein said ligand is Angiotensin II.

8. A method according to Claim 7 wherein the nuclear Overhauser effect technique is ROESY.

15   9. A method according to Claim 1 wherein fluorescence compatible environment is selected from the group consisting of micelles, lipid bilayers, and solvents having a dielectric constant of about 40 or less.

20   10. A method according to Claim 1 wherein said nuclear Overhauser effect technique is NOESY.

11. A method according to Claim 1 wherein said nuclear magnetic resonance spectroscopy employing the nuclear Overhauser effect is conducted in a 25   receptor-simulating environment.

-117-

12.   A method according to Claim 1 wherein
said ligand is complementary to a membrane bound
biologically active receptor.

13.   A method according to Claim 12 wherein
said nuclear magnetic resonance spectroscopy employing
the nuclear Overhauser effect is conducted in a solvent
having a dielectric constant of about 50 or less.

14.   A method according to Claim 13 wherein
said solvent is dimethylsulfoxide.

15.   A method of modelling antagonists to a
biologically active receptor based on the model
generated for a biologically active ligand
complementary to said receptor wherein said ligand has
one or more active sites based on a charge-transfer
interaction and further has a known structural formula
which method comprises the steps of:
        a)   creating a three-dimensional spatial
model for said ligand by
                i)   determining the presence of charge-
                      transfer interaction(s) in said
                      ligand from fluorescence analysis
                      of said ligand in a fluorescence
                      compatible environment;
               ii)   determining the chemical groups
                      involved in said charge-transfer
                      interaction(s); and
              iii)   resolving remaining aspects of the
                      ligand's three-dimensional
                      conformation by obtaining
                      conformational information relative
                      to th active site(s) from nucl ar

-118-

magnetic resonance spectroscopy
employing the nuclear Overhauser
effect providing that when the
nuclear Overhauser effect technique
employed in this step is NOESY,
then the molecular weight of said
ligand is either less than about 500
or greater than about 2000; and

b)   identifying a compound having a three-
dimensional structure sufficiently similar to said
ligand so as to be complementary to said receptor and
wherein at least one of the charge-transfer
interactions in said compound has been compromised.

16.   A method according to Claim 15 wherein
said biologically active ligand is the naturally
occurring biologically active ligand complementary for
said receptor.

17.   A method according to Claim 15 wherein
said biologically active ligand has only one charge-
transfer interaction.

18.   A method according to Claim 16 wherein
said naturally occurring biologically active ligand is
selected from the group consisting of Angiotensin II,
oxytocin and vasopressin.

19.   A method according to Claim 18 wherein
said nuclear overhauser effect is ROESY.

20.   A method according to Claim 15 wherein
said biologically active receptor is a membrane bound
rec ptor.

-119-

21.   A method of modelling agonists to a
biologically active receptor based on the model
generated for a biologically active ligand
complementary to said receptor wherein said ligand has
one or more active sites based on a charge-transfer
interaction and further has a known structural formula
which comprises the steps of:

a)   creating a three-dimensional spatial
model for said ligand by

i) determining the presence of charge
transfer interaction(s) in said
ligand from fluorescence analysis
of said ligand in a fluorescence
compatible environment; and

ii) determining the chemical groups
involved in said charge-transfer
interaction(s); and

iii) resolving remaining aspects of the
ligand's three-dimensional
conformation by obtaining
conformational information relative
to the active site(s) from nuclear
magnetic resonance spectroscopy
employing the nuclear Overhauser
effect providing that when the
nuclear Overhauser effect technique
employed in this step is NOESY,
then the molecular weight of said
ligand is either less than about 500
or greater than about 2000; and

b)   identifying a compound having a three-
dimensional structure sufficiently similar to said
ligand so as to be complementary to said receptor and

-120-

wh rein the charge-transfer interaction(s) in said
compound has (have) not been compromised.

22. A method according to Claim 21 wherein
said biologically active ligand is the naturally
occurring biologically active ligand complementary to
said receptor.

23. A method according to Claim 22 wherein
said naturally occurring biologically active ligand has
only one charge-transfer interaction.

24. A method according to Claim 23 wherein
said naturally occurring biologically active ligand is
selected from the group consisting of Angiotensin II,
oxytocin and vasopressin.

25. A method according to Claim 24 wherein
said nuclear Overhauser effect is ROESY.

26. A method according to Claim 21 wherein
said biologically active receptor is a membrane bound
receptor.

27. A three-dimensional model for
Angiotensin II as depicted in FIGURE 6.

28. A method for modelling an antagonist to
Angiotensin II which comprises creating a compound
having a three-dimensional structure sufficiently
similar to the assignment for Angiotensin II defined in
Claim 27 so as to be complementary to the biologically
active receptor for Angtiotensin II and wherein the

-121-

charge-transfer interaction in said compound has been compromised.

29. A method for modelling an agonist to Angiotensin II which comprises creating compounds having a three-dimensional structure sufficiently similar to the assignment for Angiotensin II defined in Claim 27 so as to be complementary to the biologically active receptor for Angiotensin II and wherein the charge-transfer interaction in said compound has not been compromised.

30. A three-dimensional model for receptor bound Angiotensin II as depicted in FIGURE 8B.

31. A method for determining the presence of charge-transfer interaction(s) in the tertiary structure of a biologically active ligand complementary to a biologically active receptor which comprises conducting fluorescence analysis of said ligand in a fluorescence compatible environment.

32. A method according to Claim 31 wherein said fluorescence compatible environment comprises micelles, lipid bilayers, and solvents having a dielectric constant of less than about 40.

33. A method according to Claim 31 wherein said biologically active ligand has only one charge-transfer interaction.

34. A method according to Claim 33 wherein said biologically active ligand is the naturally occurring biologically active ligand.

-122-

35. A m thod according to Claim 34 wherein said naturally occurring biologically active ligand is selected from the group consisting of Angiotensin II, oxytocin and vasopressin.

36. A method according to Claim 31 wherein said biologically active receptor is a membrane bound receptor.

37. A compound of the formula

$$R_3-C \underline{\quad\quad} C-R_4$$
$$R-HC-N \bigcirc N$$
$$R_1 \quad\quad C$$
$$CH\ R_2$$
$$(CH_2)_2$$
$$CH_3$$
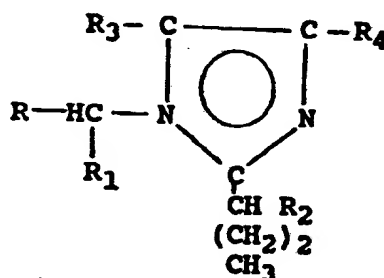
wherein R is selected from the group consisting of phenyl para substituted with a substituent selected from the group consisting of carboxyl or a pharmaceutically acceptable salt thereof, sulfate, and trifluoromethylsulfonamido, and $-NHC(O)R_5$ wherein R_5 is phenyl ortho substituted with a substituent selected from the group consisting of carboxyl or a pharmaceutically acceptable salt thereof, sulfate, and trifluoromethylsulfonamido; wherein R_1, R_2, R_3 and/or R4 provide one or more of the following charges:

i) a cationic charge at a direction left of the center of the imidazole ring at a distance of about 7 ± 1.5 Angstroms;

ii) an anionic charge at a direction right of the center of the imidazole ring at a distanc of about 2.5 ± 0.5 Angstroms;

-123-

iii)   an anionic charge at a direction left
       of the center of the imidazole ring at a
       distance of about 10 ± 2 Angstroms; and

iv)   a cationic charge at a direction left
       of the center of the imidazole ring at a
       distance of about 12 ± 2.5 Angstroms;
       providing that when any of R_1, R_2, R_3, and
       R_4 are not providing such an ionic charge
       then R_1 is hydrogen, R_2 is hydrogen, R_3 is
       either hydroxymethyl, $-CH_2-O-CH_3$,
       $-CH_2C(O)OCH_3$ or $-C(O)OCH_3$ and R_4 is
       fluorine or chlorine, further providing
       that said compound contains no more than
       one charge for each of i), ii), iii) and
       iv), and still further providing that
       the placement of such charges left
       and/or right of the imidazole ring is as
       defined in Formula I and II of this
       application.

38.   A pharmaceutical composition which
comprises a pharmaceutically acceptable carrier and an
effective amount to control hypertension in a mammal in
need of such treatment of a compound defined in
Claim 37.

39.   A pharmaceutical composition which
comprises a pharmaceutically acceptable carrier and an
effective amount to control congestive heart failure in
a mammal in need of such treatment of a compound
defined in Claim 37.

-124-

40.   A method of controlling hypertension in
a mammal in need of such treatment by administering
either orally or parenterally a pharmaceutical
composition as defined in Claim 38.

5   41.   A method of controlling congestive heart
failure in a mammal in need of such treatment by
administering either orally or parenterally a
pharmaceutical composition as defined in Claim 39.

42.   A compound of the formula

$$R_3 - C \underline{\hspace{2cm}} C\text{-}R_4$$
$$R\text{-}HC - N \qquad N$$
$$R_1 \qquad C$$
$$CH\text{-}R_2$$
$$(CH)_2$$
$$CH_3$$
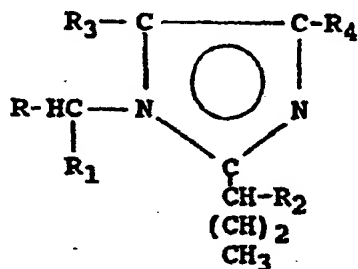
10   wherein R is selected from the group consisting of
a) phenyl para substituted with a substituent selected
from the group consisting of carboxyl or a
pharmaceutically acceptable salt thereof, sulfate, and
trifluoromethylsulfonamido, and b) -NHC(O)R_5 wherein R_5
15   is phenyl ortho substituted with a substituent selected
from an acidic group consisting of carboxyl or a
pharmaceutically acceptable salt thereof, sulfate, and
trifluoromethylsulfonamido, wherein either R_1 or R_3 but
not both provides a cationic charge left of the center
20   of the imidazole ring at a distance of about 7 ± 1.5
Angstroms providing that when R_3 provides such a charge
then Rl is hydrogen and further providing that when R_1
provides such a charge then R_3 is hydroxymethyl,

-125-

$-CH_2OCH_3$, $-CH_2CO_2CH_3$ or $-CO_2CH_3$ further wherein either R_2 or R_4 but not both provides an anionic charge right of the center of the imidazole ring at a distance of about 2.5 ± 0.5 Angstroms providing that when R_2 provides such a charge, then R4 is either fluorine or chlorine and further providing that when R_4 provides such a charge, then R_2 is hydrogen and still further providing that the placement of charges left and/or right of the imidazole ring is as defined in Formula I and II of this application.

43.   A compound as defined in Claim 40 wherein R_1 is hydrogen and R_3 is selected from the group consisting of $-(CH_2)_4-NH_2$, $-CHOH-(CH_2)_3-NH_2$, and $-(CH_2)_4-Asp-Arg-NH_2$.

44.   A compound as defined in Claim 40 wherein R_3 is hydroxymethyl, $-CH_2OCH_3$, $-CH_2CO_2CH_3$ or $-CO_2CH_3$ and R_1 is selected from the group consisting of $-(CH_2)_3-NH_2$ and $-(CH_2)_3-Asp-Arg-NH_2$.

45.   A compound as defined in Claim 40 wherein R_2 is hydrogen and R_4 is $-(CH_2))_3-C(O)OH$.

46.   A compound as defined in Claim 40 wherein R_4 is either hydrogen or chlorine and R_2 is $-(CH_2)_3-C(O)OH$.

47.   A pharmaceutical composition which comprises a pharmaceutically acceptable carrier and an effective amount to control hypertension in a mammal in need of such treatment of a compound as defined in Claim 42.

-126-

48. A pharmac utical composition which comprises a pharmaceutically acceptable carrier and an effective amount to control congestive heart failure in a mammal in need of such treatment of a compound as defined in Claim 42.

49. A method of controlling hypertension in a mammal in need of such treatment which comprises administering either orally or parenterally a pharmeceutical composition defined in Claim 47.

50. A method of controlling congestive heart failure in a mammal in need of such treatment which comprises administering either orally or parenterally a pharmeceutical composition defined in Claim 48.

51. A compound of the formula:

$$R^{4A} - CH - \alpha \bigcirc \begin{matrix} R^3 & R^2 \\ \epsilon & \delta \\ & \gamma \end{matrix} - R^2 \text{ or } R^3$$

$$\begin{matrix} | \\ R^{4B} \end{matrix} \quad \beta$$

$$CH - R^{1B}$$

$$R^{1A}$$
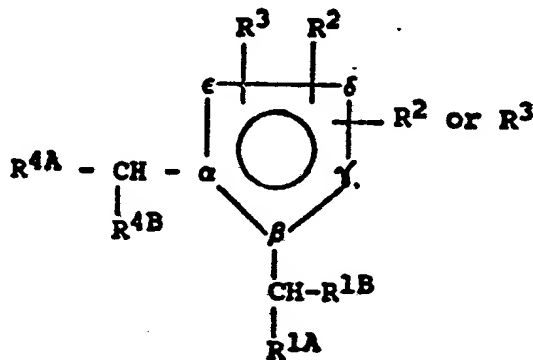
wherein α, β, γ, δ, and ϵ are C, N, O or S with the provisos that (a) the ring contains at least one C atom and one N atom and (b) attachment of R groups is to C or N;
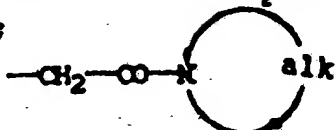
-127-

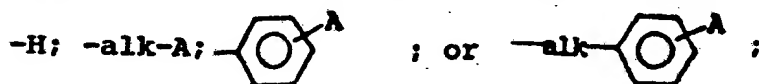R^{1A}, which mimics the structure in angiotensin of

 includes the following:

-alk; -O-alk; -alk-O-alk; $-CH_2-CO-NH_2$; $-CH_2-CO-NH-alk$; $-CH_2-CO-N(alk)_2$;

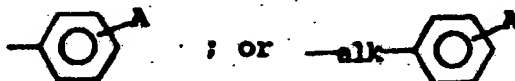$-CH_2-CO-AA-NH_2$; or $-CH_2-CO-AA-Phe$, wherein AA is an amino acid;

R^{1B}, which provides a spacer arm terminating in a mimic of the C-terminal carboxylate group of angiotensin II, includes the following:

-H; -alk-A; ; or ;

R^2, which provides steric and/or electronic properties and/or a spacer arm terminating in an acid group, includes the following: -H, -halide; -alk; -O-alk; $-NO_2$; $-CF_3$; -CN; -alk-A; -A;

 ; or ;

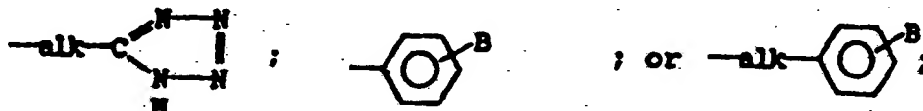R^3, which provides steric and/or electronic properties and/or a mimetic of the tyrosine hydroxyl group of angiotensin II in its "charge relay" conformation, or a spacer arm terminating in a mimic of the N-terminus of N-terminal dipeptide of angiotensin-II, includes the following:

-H; -alk; -aryl; -alk-OH; -alk-halide; $-CH_2-O-alk$; $-CH_2-CN$; $-CH_2-CO_2H$; $-CH_2CO_2-alk$; -NH-CO-alk; -CO-NH-alk; -alk-B; -CH(OH)-alk-B; $-alk-Asp-Arg-NH_2$; $-CH(OH)-alk-Asp-Arg-NH_2$;
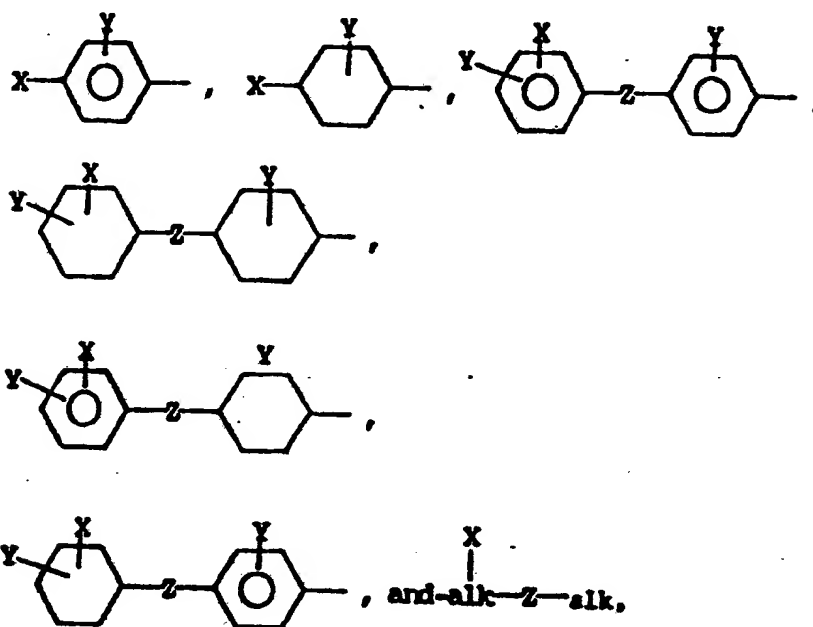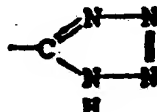
 ; ; r ;

-128-

R^{6A}, which provides a spacer arm, the relative rigidity of which is an aspect of the design, terminating in an acid group which mimics the tyrosine hydroxy groups of angiotensin II in its "receptor bound" conformation, or a spacer arm terminating in a mimic of the N-terminus or N-terminal dipeptide of angiotensin II, includes the following:

where Z is a bond, -NHCO-, -O-, -OCH$_2$-, or -CH$_2$-;
X is -CO$_2$H, -alk-CO$_2$H, -PO$_3$H, -alk-PO$_3$H, -PO$_4$H$_2$, -alk-PO$_4$H$_2$, -SH, -alk-SH, -SO$_3$H, -alk-SO$_3$H, -SO$_4$H$_2$, -alk-SO$_4$H$_2$, F$_3$C-CO-NH-, F$_3$C-SO$_2$-NH-,

or a pharmaceutically acceptable salt thereof; and
Y is -H, -halide, -NO$_2$, -O-alk, -alk, -CF$_3$, or -CN; and

-129-

R^{48}, which provides a spacer arm terminating in a mimic of the N-terminus or N-terminal dipeptide of angiotensin, includes the following:

-H, -alk-B, -alk-Asp-Arg-NH$_2$, -alk-O-alk-B,

-alk-O-alk-Asp-Arg-NH$_2$,

alk = an alkyl group having from 1 to 10 carbon atoms, a cycloalkyl group having 3-6 carbon atoms, an alkenyl group having 2-10 carbon atoms, or an alkynyl group having 2-10 carbon atoms;

halide = -F, -Cl, -Br, or -I;

A = an acid group or its pharmaceutical salt;

and   B = a basic group or its pharmaceutical salt.

52.   A compound of Claim 51 wherein when R^{1B} is H, then: (a) if the ring is imidazole either α or γ is other than N, (b) if the ring is other than imidazole either α is C or β is N, (c) R^{1A} comprises a group containing an amide, (d) R^2 comprises a group containing A, or (e) R^3 comprises a group containing B or -Asp-Arg-NH$_2$.

53.   A compound of Claim 51 wherein when R^{48} is H, then: (a) if the ring is imidazole either α or γ is other than N, (b) if the ring is other than imidazole either α is C or β is N, (c) R^{1A} comprises a group containing an amide, (d) R^2 comprises a group containing A, or (e) R^3 comprises a group containing B or -Asp-Arg-NH$_2$.

-130-

54. A compound of Claim 51 wherein the ring is imidazole, pyrole, pyrrazole, 1,2,3- and 1,2,4-triazole, tetrazole or thiazole, oxazole, thiadiazole, or oxadiazole.

5

55. A compound of Claim 54 wherein the ring is an imidazole.

56. A compound of Claim 51 wherein at least one of the substitutents R^2 or R^3, which are bonded to the ring atoms γ, δ, or ϵ is hydrogen.

10

57. A compound of Claim 51 wherein R^{1B} or R^{4B} is hydrogen.

58. A compound of the formula:
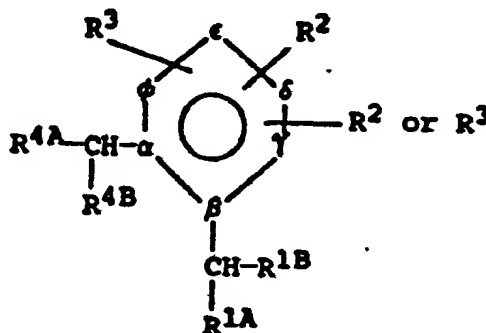
15

wherein α, β, γ, δ, ϵ, and ϕ are C, N, O or S with the provisos that (a) the ring contains at least one C atom and one N atom and (b) attachment of R groups is to C or N;

R^{1A}, which mimics the structure in angiotensin f

-131-

-CH-CO-N-CH-CO-   includes the following:

-alk; -O-alk; -alk-O-alk; -CH$_2$-CO-NH$_2$; -CH$_2$-CO-NH-alk;
-CH$_2$-CO-N(alk)$_2$;

-CH$_2$-CO-N   alk

-CH$_2$-CO-AA-NH$_2$; or -CH$_2$-CO-AA-Phe,
wherein AA is an amino acid;

R^{1B}, which provides a spacer arm terminating
in a mimic of the C-terminal carboxylate group of
angiotensin II, includes the following:

-H; -alk-A; -⟨O⟩-A   ; or -alk-⟨O⟩-A ;

R^2, which provides steric and/or electronic
properties and/or a spacer arm terminating in an acid
group, includes the following: -H, -halide; -alk;
-O-alk; -NO$_2$; -CF$_3$; -CN; -alk-A; -A;

-⟨O⟩-A   ; or -alk-⟨O⟩-A

R^3, which provides steric and/or electronic
properties and/or a mimetic of the tyrosine hydroxyl
group of angiotensin II in its "charge relay"
conformation, or a spacer arm terminating in a mimic of
the N-terminus of N-terminal dipeptide of angiotensin-
II, includes the following:
-H; -alk; -aryl; -alk-OH; -alk-halide; -CH$_2$-O-alk;
-CH$_2$-CN; -CH$_2$-CO$_2$H; -CH$_2$CO$_2$-alk; -NH-CO-alk;
-CO-NH-alk; -alk-B; -CH(OH)-alk-B; -alk-Asp-Arg-NH$_2$;
-CH(OH)-alk-Asp-Arg-NH$_2$;

-alk-C⟨N-N | N-N⟩N ;   -⟨O⟩-B   ; or -alk-⟨O⟩-B ;

-132-

R^{4A}, which provides a spacer arm, the relative rigidity of which is an aspect of the design, terminating in an acid group which mimics the tyrosine hydroxy groups of angiotensin II in its "receptor bound" conformation, or a spacer arm terminating in a mimic of the N-terminus or N-terminal dipeptide of angiotensin II, includes the following:

-133-

where Z is a bond, -NHCO-, -O-, -OCH$_2$-, or -CH$_2$-;
X is -CO$_2$H, -alk-CO$_2$H, -PO$_3$H, -alk-PO$_3$H, -PO$_4$H$_2$, -alk-PO$_4$H$_2$, -SH, -alk-SH, -SO$_3$H, -alk-SO$_3$H, -SO$_4$H$_2$, -alk-SO$_4$H$_2$, F$_3$C-CO-NH-, F$_3$C-SO$_2$-NH-,
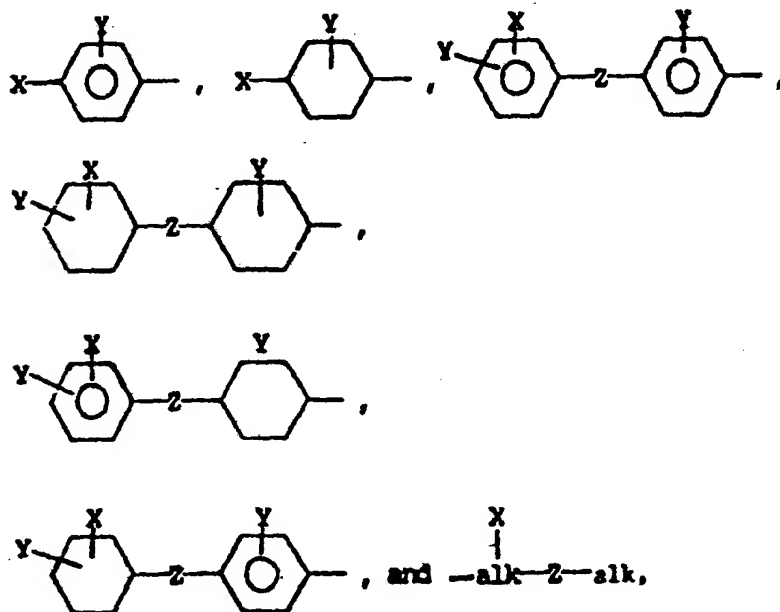

5   or a pharmaceutically acceptable salt thereof; and
Y is -H, -halide, -NO$_2$, -O-alk, -alk, -CF$_3$, or -CN; and
R^{48}, which provides a spacer arm terminating
in a mimic of the N-terminus or N-terminal dipeptide of
angiotensin, includes the following:
10   -H, -alk-B, -alk-Asp-Arg-NH$_2$, -alk-O-alk-B,
-alk-O-alk-Asp-Arg-NH$_2$,

alk = an alkyl group having from 1 to 10
carbon atoms, a cycloalkyl group having 3-6 carbon
atoms, an alkenyl group having 2-10 carbon atoms, or an
15   alkynyl group having 2-10 carbon atoms;
halide = -F, -Cl, -Br, or -I;
A = an acid group or its pharmaceutical salt;
and   B = a basic group or its pharmaceutical salt.

59. A compound of Claim 58 wherein when R^{18}
20   is H, then: (a) if the ring is imidazole either α or γ
is other than N, (b) if the ring is other than
imidazole either α is C or β is N, (c) R^{1A} comprises a
group containing an amide, (d) R^2 comprises a group
containing A, or (e) R^3 comprises a group containing B
25   or -Asp-Arg-NH$_2$.

SUBSTITUTE SHEET

-134-

60.　A compound of Claim 58 wherein when R^{4B} is H, then: (a) if the ring is imidazole either α or γ is other than N, (b) if the ring is other than imidazole either α is C or β is N, (c) R^{1A} comprises a
5　group containing an amide, (d) R^2 comprises a group containing A, or (e) R^3 comprises a group containing B or $-Asp-Arg-NH_2$;

61.　A compound of Claim 56 wherein at least one of the substitutents R^2 or R^3, which are bonded to
10　the ring atoms γ, δ, or ϵ is hydrogen.

62.　A compound of the formula:
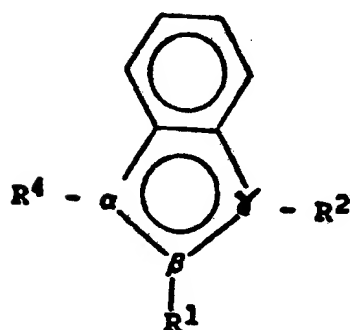
(I)
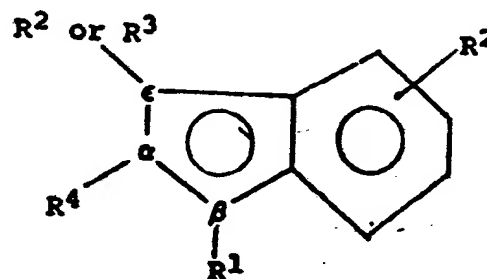
(II)
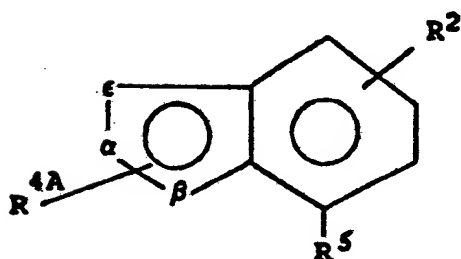
(III)

SUBSTITUTE SHEET

wherein α, β, and γ are C or N, with the provisos that
only one N atom is substituted;
R¹ is -CH(R^{1A})(R^{1B}) wherein

    R^{1A}, which mimics the structure in
angiotensin of

includes the following:

-alk; -O-alk; -alk-O-alk; -CH₂-CO-NH₂; -CH₂-CO-NH-alk;
-CH₂-CO-N(alk)₂;
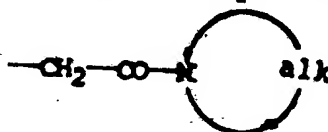
-CH₂-CO-AA-NH₂; or -CH₂-CO-AA-Phe,
wherein AA is an amino acid, azetidine-carboxylic acid,
pipecolic acid, nipecotic acid, glycine, alanine,
sarcosine, or N-methyl-alanine;

    R^{1B}, which provides a spacer arm terminating
in a mimic of the C-terminal carboxylate group of
angiotensin II, includes the following:

-H; -alk-A; ; or ;

with the proviso that when R^{1B} is H then (a) R^{1A}
comprises a group containing an amide, or (b) R¹ is on
an N, or (c) R⁴ is on a C;

    R², which provides steric and/or electronic
properties and/or a spacer arm terminating in an acid
group, includes the following: -H, -halide; -alk;
-O-alk; -NO₂; -CF₃; -CN; -alk-A; -A;

 ; or

    R³, which provides steric and/or electronic
properties and/or a mimetic of the tyrosine hydroxyl

group of angiotensin II in its "charge relay"
conformation, or a spacer arm terminating in a mimic of
the N-terminus of N-terminal dipeptide of angiotensin-
II, includes the following;

-H; -alk; -aryl; -alk-OH; -alk-halide; $-CH_2-O-alk$;
$-CH_2-CN$; $-CH_2-CO_2H$; $-CH_2CO_2-alk$; -NH-CO-alk;
-CO-NH-alk; -alk-B; -CH(OH)-alk-B; $-alk-Asp-Arg-NH_2$;
$-CH(OH)-alk-Asp-Arg-NH_2$;

R^4 is $-CH(R^{4A})(R^{4B})$ wherein

R^{4A}, which provides a spacer arm, the
relative rigidity of which is an aspect of the design,
terminating in an acid group which mimics the tyrosine
hydroxy groups of angiotensin II in its "receptor
bound" conformation, or a spacer arm terminating in a
mimic of the N-terminus or N-terminal dipeptide of
angiotensin II, includes the following:

-137-

where Z is a bond, -NHCO-, -O-, -OCH$_2$-, or -CH$_2$-;
X is -CO$_2$H, -alk-CO$_2$H, -PO$_3$H, -alk-PO$_3$H, -PO$_4$H$_2$, -alk-PO$_4$H$_2$, -SH, -alk-SH, -SO$_3$H, -alk-SO$_3$H, -SO$_4$H$_2$, -alk-SO$_4$H$_2$, F$_3$C-CO-NH-, F$_3$C-SO$_2$-NH-,

or a pharmaceutically acceptable salt thereof; and

Y is -H, -halide, -NO$_2$, -O-alk, -alk, -CF$_3$, or -CN; and

R^{4B}, which provides a spacer arm terminating in a mimic of the N-terminus or N-terminal dipeptide of angiotensin, includes the following:

-H, -alk-B, -alk-Asp-Arg-NH$_2$, -alk-O-alk-B, -alk-O-alk-Asp-Arg-NH$_2$,

R^5 is the same as R^1 defined above;

alk = an alkyl group having from 1 to 10 carbon atoms, a cycloalkyl group having 3-6 carbon atoms, an alkenyl group having 2-10 carbon atoms, or an alkynyl group having 2-10 carbon atoms;

halide = -F, -Cl, -Br, or -I;

A = an acid group or its pharmaceutical salt;

and   B = a basic group or its pharmaceutical salt.
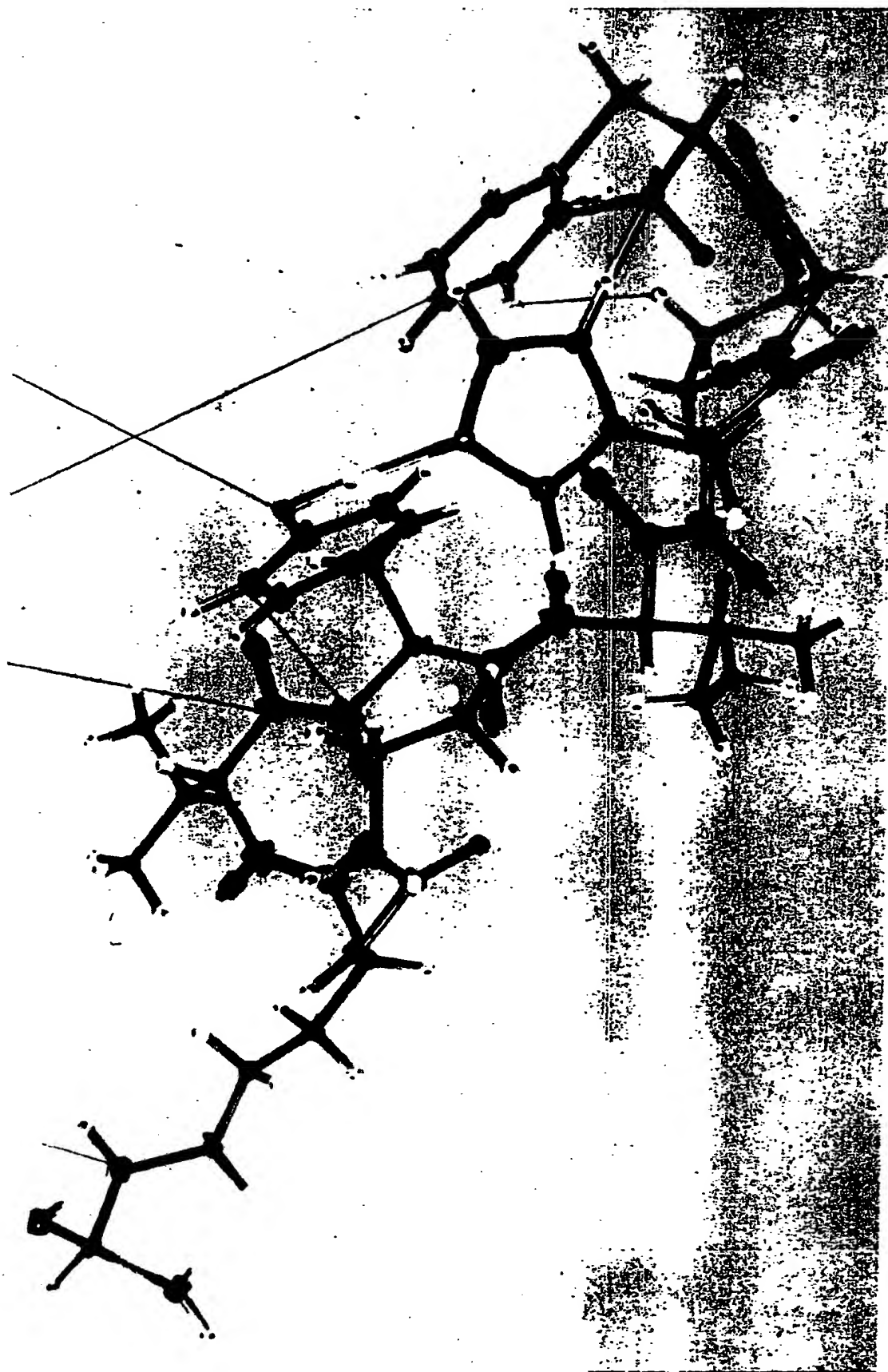
FIG._1

FIG._2A

FIG._2B

FIG._2C

SUBSTITUTE SHEET

3/10

BI

FIG._3A

BABI
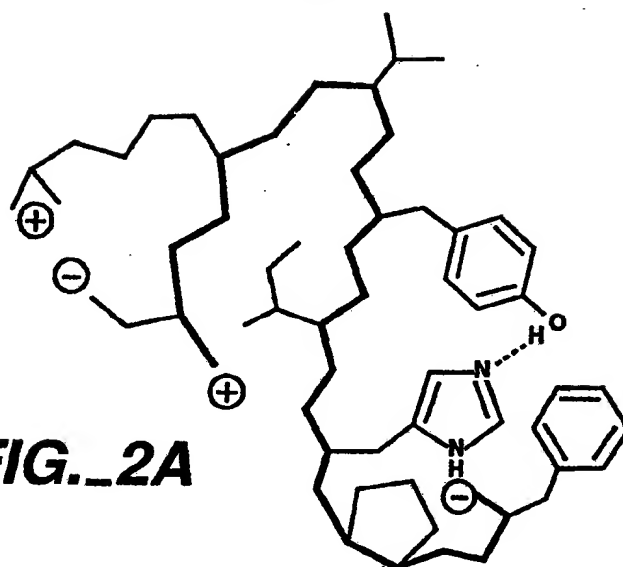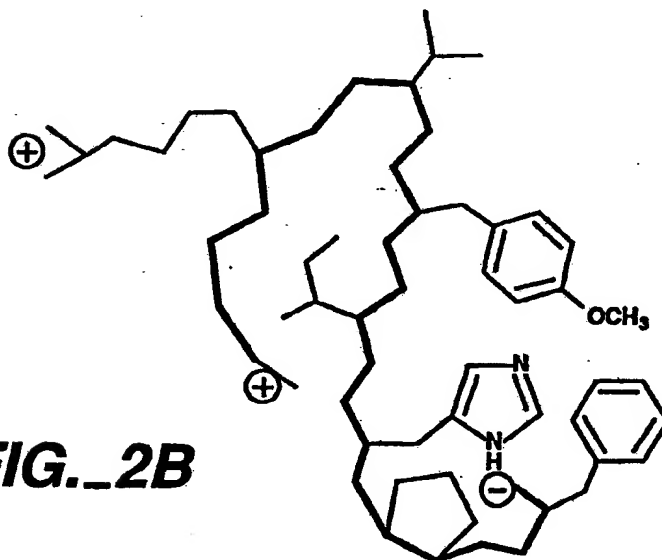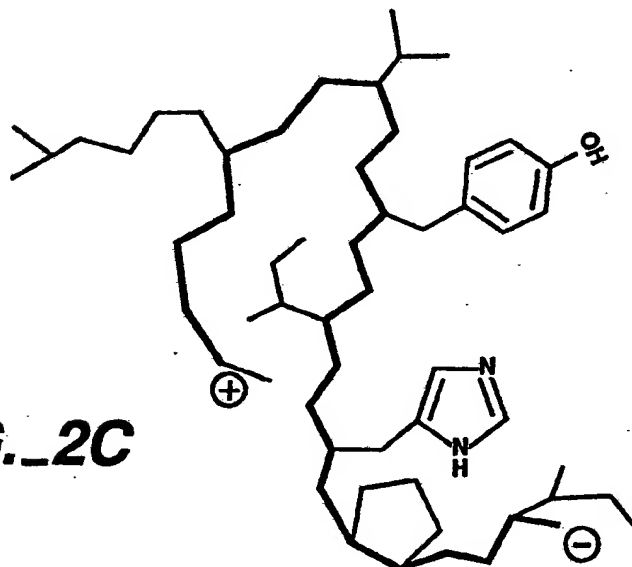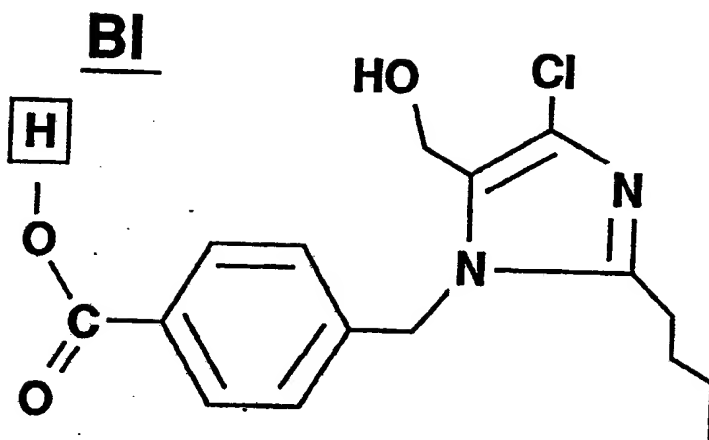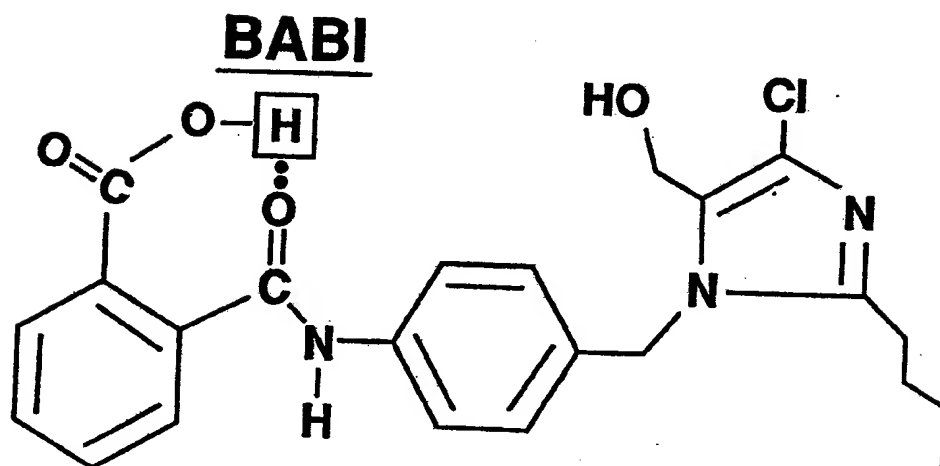
FIG._3B

SUBSTITUTE SHEET

4/10

FIG._4A

FIG._4B

6/10

FIG._5A

FIG._5B

SUBSTITUTE SHEET

FIG._6

8/10

FIG._7A

FIG._7B

10/10

FIG._7C